FORM PTO-1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO (If known, see 37 CFR 1.5)
410015	09/297519

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR97/02022	November 10, 1997	November 15, 1996

TITLE OF INVENTION: NOVEL POLYMERIC COMPLEXES FOR THE TRANSFECTION OF NUCLEIC ACIDS, WITH RESIDUES CAUSING THE DESTABILIZATION OF CELL MEMBRANES

APPLICANT(S) FOR DO/EO/US: MIDOUX et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [ ] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Verified Statement Claiming Small Entity Status (Small Business Concern); Drawings (9 sheets); PCT/IPEA/416; PCT/IPEA/409; PCT/IPEA/408;

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
	PCT/FR97/02022	410.015

17. [X] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO..... $830.00	$ 970.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) $640.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..... $710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2)) paid to USPTO..... $950.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... $90.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 970.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	19 -20 =	0	X $22.00	$	
Independent Claims	4 -3 =	1	X $78.00	$ 78.00	
Multiple dependent claims(s) (if applicable)			+ $230.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1048.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$ 524.00	
SUBTOTAL =				$ 524.00	
Processing fee of $130.00 for furnishing the English translation later the [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 524.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$ 40.00	
TOTAL FEES ENCLOSED =				$ 564.00	
				Amount to be: refunded	$
				charged	$

a. [X] A check in the amount of $564.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2275. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Charles A. Muserlian
Bierman, Muserlian and Lucas
600 Third Avenue
New York, NY 10016

Charles A. Muserlian
SIGNATURE

Charles A. Muserlian
NAME

19,683
REGISTRATION NUMBER

Form PTO-1390 (REV 5-93)

PTO/SB/10 (11-90)

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(c))--SMALL BUSINESS CONCERN	Docket Number (Optional) 110.015

Applicant or Patentee: Patrick MIDOUX and Michel MONSIGNY
Serial or Patent No.: PCT/FR97/02022
Filed or Issued: November 10, 1997
Title: NOVEL POLYMERIC COMPLEXES FOR THE TRANSFECTION OF NUCLEIC ACIDS, WITH RESIDUES CAUSING THE DESTABILIZATION OF CELL MEMBRANES

I hereby declare that I am

☐ the owner of the small business concern identified below;
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN I.D.M. IMMUNO-DESIGNED MOLECULES
ADDRESS OF SMALL BUSINESS CONCERN 172, rue de Charonne, F-75011, Paris, France

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in:

☐ the specification filed herewith with title as listed above.
☒ the application identified above.
☐ the patent identified above.

If the rights held by the above identified small business concern are not exclusive, each individual concern or organization having rights in the invention must file separate verified statements averring to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:

☒ no such person, concern, or organization exists.
☐ each such person, concern or organization is listed below.

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Jean-Loup ROMET-LEMONNE
TITLE OF PERSON IF OTHER THAN OWNER
ADDRESS OF PERSON SIGNING 172, rue de Charonne, F-75011, Paris, France
SIGNATURE J.L. Romet-Lemonne DATE April 16, 1999

Our Ref.: 410.015

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:
Patrick MIDOUX et al	:
PCT/FR97/02022	:
Serial No.:	: PCT Date: November 10, 1997
Filed: Concurrently Herewith	:
For: NOVEL...CELL MEMBRANES	:
	600 Third Avenue
	New York, NY 10016

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend this application as follows:

IN THE CLAIMS:

Claims 4 and 7, line 1 of each, cancel "one of the claims 1 to 3" and insert --claim 1--.

Claim 5, line 1, cancel "any one of claims 1 to 3" and insert --claim 1--.

Claims 6 and 9, line 1 of each, cancel "one of claims 1 to 5" and insert --claim 1--.

Claim 10, line 1, cancel "one of claims 1 to 6" and insert --claim 1--.

Claim 11, line 1, cancel "one of claims 1 to 7" and insert --claim 1--.

Cancel claims 13 and 18 and add the following claims:

--20. A polymeric conjugate of claim 12 with a complex of claim 2--.

--21. In the method of transfecting cells with a high efficiency with a gene, the improvement comprising using the complex of claim 6.--

Claim 15, cancel lines 1 and 2 and insert --The method of claim 21 wherein the cells are selected from the group consisting of--.

line 17, insert at the end --and--.

line 18, insert at the end --.--

Cancel line 19.

Claim 16, line 2, cancel "any one of claims 1 to 11" and insert --claim 1--.

Claim 17, line 2, cancel "any one of claims 1 to 11" and

insert --claim 1--.

line 3, cancel "one of claims 12 or 13" and insert --claim 12--.

Claim 19, line 2, cancel "one of claims 12 or 13", such as" and insert --claim 12 with a--.

line 11, cancel "one of claims 1 to 11" and insert --claim 1--.

REMARKS

The amendment is submitted to remove multiple dependent claims from the application.

Respectfully submitted,
BIERMAN, MUSERLIAN AND LUCAS

Charles A. Muserlian, #19,683
Attorney for Applicant(s)
Tel. # (212) 661-8000

CAM:sd
Enclosure: Return Receipt Postcard

NOVEL POLYMERIC COMPLEXES FOR THE TRANSFECTION OF NUCLEIC ACIDS, WITH RESIDUES CAUSING THE DESTABILISATION OF CELL MEMBRANES.

The invention relates to new complexes of nucleic acids and polymer substituted by residues which cause destabilization of cell membranes.

The introduction of a foreign gene into a cell is the basis of genetic treatment. The transfer of genes can be achieved using either a modified viral material (vaccine virus, retrovirus, adenovirus or herpes virus) or using non-viral vectors (cationic lipids, liposomes). The former, although effective, have safety problems. As regards the latter, the effectiveness is greatly reduced in the presence of serum, and as a result their use is restricted to *in vitro* or *ex vivo*.

Polylysine, which can form stable electrostatic complexes with a plasmid DNA is the basis for development of non-viral vectors for transfer of genes in animal cells.

Complexes of DNA and unsubstituted polylysine generally are not effective for transfection of cells because of the very high stability of the complexes (and therefore weak dissociation and salting out of the DNA) under physiological conditions as a consequence of a very high co-operativity of polycation-polyanion interactions.

The transfection efficiency can be improved if the number of charges present on the polypeptide is decreased in order to reduce the interactive forces between the DNA and the polylysine. For example, if 40% of the ε-NH_3^+ functions of the lysine residues of the polylysine are partly neutralized by polyhydroxyalkanoyl derivatives, such as δ-gluconolactone, the DNA/partly gluconylated polylysine complexes are more effective than DNA/polylysine complexes in transfection of cells.

The polylysine can be substituted by specific receptor ligands which are present on the surface of cells and are capable of inducing specific endocytosis of complexes with a plasmid DNA by target cells.

Conjugates obtained by substituting polylysine by asialoorosomucoid, transferrin, insulin, immunoglobulin and growth factors have been proposed as plasmid guide vectors. However, these protein ligands render the complexes highly immunogenic.

The polylysine can be substituted by low molecular weight ligands which are less immunogenic than the osides and oligosides recognized by specific membrane receptors (membrane lectins) on the surface of target cells. Glycosylated polylysine has been proposed

as non-viral vectors perfectly defined for transfer of genes.

Numerous animal cells have membrane lectins which recognize oligosides of various structures and which induce endocytosis of their ligands. For example, the membrane lectin of cells of the hepatic parenchyma recognize glucidic structures carrying a galactose residue in the terminal position, which is the case for the desialylated serum glycoproteins. The specificity of membrane lectins depends on the cell type and the state of differentiation of the cells.

The transfection efficiency of DNA/glycosylated polylysine complexes depends on the level of substitution of the polylysine by osides: The most effective transfections are obtained if 30 to 40% of the ε-NH_3^+ functions of the lysine residues of the polylysine are substituted by mono- or disaccharides.

In French Patent no. 2,719,316, it has been shown that the use of partly gluconylated polylysine carrying an already reduced number of positive charges allows the number of osides required for bonding on the polymer to obtain a good transfection efficiency of DNA/glycosylated and gluconylated polylysine complexes to be decreased by a factor of 5 to 10. The use of partly gluconylated polylysine allows the solubility of complexes to be increased and their size to be reduced to about 50 nm.

The transportation of plasmids by non-viral vectors which can be recognized specifically by compounds of the plasma membrane of cells is dependent on a step which imitates the mechanism of entry of viral genetic material into a cell. In all the cases described, the DNA/polycationic polymer complexes are carried into endocytosis vesicles, into endosomes and probably into other deeper intracellular compartments removed from the plasma membrane.

The transmembrane passage of plasmid DNA is consequently a critical stage with respect to the release of the said DNA into the cytosol for its passage into the nucleus where the gene will be expressed.

In all the cases described, transmembrane passage auxiliaries are used to promote passage of the DNA into the cytosol. These are:

- chloroquine
- defective adenoviruses
- permeabilizing and/or fusiogenic peptides

a) Chloroquine is a weak base used in an amount of 50 μM or 100 μM in culture *in*

vitro and for some cells these concentrations are toxic. Chloroquine, which permeates, crosses the membrane and accumulates in the acid compartments because it carries amines of low pK which capture protons; protonated chloroquine is cationic and less permeating. Acidification of endosomes and lysosomes is caused by a membrane enzyme which injects H^+ from cytosol into vesicles; to re-establish electroneutrality, this proton accumulation is accompanied by an entry of chloride ions Cl^-. To the extent that chloroquine accumulates, protons and chlorides also accumulate, which causes an increase in the intravesicular ionic force, which induces the arrival of water, resulting in swelling of the vesicles and their destabilization. The intracellular concentration of chloroquine can be more than 100 times higher than its concentration in the medium after a few hours. It can thus exceed 10 mM. This phenomenon is comparable to that which occurs in persons who use a daily dose of 300 mg chloroquine per day. After a few days, the plasma concentration is about 0.7 μM and the tissue concentration is 200 to 700 times higher, that is to say 140 to 500 μM. Inside the cells, the acid compartments can reach concentrations several tens of times higher. It is furthermore known that chloroquine concentrations of 10 mM (concentration obtained a few hours after having used an initial chloroquine concentration of 100 μM) promote dissociation of DNA/polylysine complexes.

Chloroquine in combination with DNA/polylysine complexes in gene transfer can be used only in applications *in vitro* or *ex vivo*, because of its toxicity and its rapid dilution after injection into the individual. In fact, *in vivo*, to achieve the high concentrations mentioned above, several days are necessary. It has thus been found *in vitro* that in cells pretreated with chloroquine, expression of the transferred genes was very low. In addition, if the chloroquine is added more than three hours after the incubation of the cells in the presence of the complexes, the transfection is very low. For these reasons chloroquine, which is a very good auxiliary *in vitro*, is not effective *in vivo*.

b) The fusiogenic properties of defective adenovirus particles in an acid medium are used to promote passage of DNA into the cytosol from endocytosis vesicles. Adenoviruses have fusion proteins which are active in a slightly acid medium. Defective adenoviruses can be used in the free form or bonded to DNA/polylysine complexes.

However, the use of even defective viral particles presents safety problems. Adenoviruses induce a very strong immune response after injection with the complexes.

c) Peptides which are permeabilizing and/or fusiogenic in a slightly acid medium are

used as auxiliaries to promote passage of DNA into the cytosol. These are mainly peptides of 20 amino acids derived from virus fusion proteins, such as, for example, the *N*-terminal peptide of the sub-unit HA2 of the haemagglutinin of the influenza virus, or synthetic peptides, such as GALA, an oligomer containing several recurring units of Glu-Ala-Leu-Ala. These peptides are most often used in the free form (that is to say not covalently bonded) with the DNA/polylysine complexes. The efficiency of peptides is greatly reduced in the presence of serum in the cell culture medium, which restricts their use to experiments *in vitro* or to *ex vivo*. Some peptides covalently bonded to DNA/polylysine complexes are still effective in promoting transmembrane passage of DNA, while others lose their permeabilizing power after bonding.

It is furthermore known that there are other molecules which are capable of destabilizing membranes, and in particular molecules containing the imidazole nucleus of histidine (pK = 6.04), which, by being protonated in a slightly acid medium, become cationic. Polyhistidine has fusiogenic and permeabilizing properties with respect to lipid bilayers. At pH <6, polyhistidine adopts an α-helical structure (Norland K.S. *et al.* (1963) Biopolymers 1:277-278; Beychok S. *et al.* (1965) J. Amer. Chem. Soc. 87:3990-3991). It has been shown that in a slightly acid medium polyhistidine is a polycation which aggregates negatively charged liposomes and induces their fusion (Wang C.-Y. and Huang L. (1984) Polyhistidine mediates an acid-dependent fusion of negatively charged liposomes. Biochemistry 23:4409-4416; Uster P.S. and Deamer D.W. (1985) pH-dependent fusion of liposomes using titrable polycations. Biochemistry 24:8-14).

It is known that a synthetic polymer (cetylacetyl(imidazol-4-ylmethyl)polyethyleneimine) induces the fusion of liposomes at a slightly acid pH (Oku N. *et al.*. (1987) Low pH induced membrane fusion of lipid vesicles containing proton-sensitive polymer. Biochemistry 26:8145-8150).

It is also known that a neutral water-soluble polymer substituted by histidyl residues (used instead of polyhistidine, which is very poorly soluble in an aqueous medium) interacts with a polyanion, such as polyaspartic acid, only in a slightly acid medium and is capable of permeabilizing the plasma membrane of cells in a flow cytometry test using ethidium bromide as a marker (Midoux *et al.*, 1995).

Preliminary results have shown that polyhistidine (very poorly soluble in an aqueous medium at neutral pH) cannot be used to transfect cells because, since it is not a polycation at

neutral pH, it is not capable of forming with DNA stable complexes of sufficient solubility to be used at neutral pH, in particular at pH 7.4, the pH of plasma.

The invention relates to new complexes of nucleic acid and substituted polymer which are capable of transfecting several types of cells.

The invention relates to a new type of cationic polymer comprising, in addition to positive charges of the polymer, substituents which promote transmembrane passage of the nucleic acid transported and, where appropriate, substituents which act as recognition signals. The substituents which promote transmembrane passage are bonded to the polymer and are derivatives which are not cationic in a slightly alkaline medium but become so in a neutral medium and in an acid medium.

The invention relates to new complexes of nucleic acid and substituted polymer which are capable of promoting transmembrane passage of DNA after endocytosis of the complexes.

The invention relates to new complexes of nucleic acid and substituted polymer having no recognition signals recognized by membrane receptors on the surface of cells.

The invention relates to new complexes of nucleic acid and substituted polymer also having recognition signals recognized by membrane receptors on the surface of cells, conferring a selective transfection character with respect to various cell types.

The invention relates to a process for specific transfection *in vitro* and *in vivo*.

The invention relates to new conjugates of substituted polylysine having no recognition signals recognized by membrane receptors on the surface of cells, which are capable of being complexed with a nucleic acid for the purpose of transfection of a cell.

The invention also relates to new conjugates of polylysine also having recognition signals recognized by membrane receptors on the surface of cells, which are capable of being complexed with a nucleic acid for the purpose of selective transfection of a cell.

The advantage of the invention is that these new complexes of nucleic acid and polymer are capable of transfecting cells in the absence of transmembrane passage auxiliaries (chloroquine or permeabilizing and/or fusiogenic peptides). These are weakly basic groupings which can be protonated (cation) in a slightly acid medium and are bonded to the polymer and play the role of transmembrane passage auxiliaries.

The advantage of the invention is that these new complexes of nucleic acid and substituted polymer are as effective as or, without transmembrane passage auxiliaries, more effective than the complexes of nucleic acid and polymer which is unsubstituted or

substituted by agents which reduce the number of charges on the polymer (and therefore its interaction with the nucleic acid) in the presence of transmembrane passage auxiliaries.

In the case of chloroquine and permeabilizing and/or fusiogenic peptides, the latter are small molecules which diffuse rapidly if they are not covalently bonded to complexes of nucleic acid and substituted polymer.

The advantage of the invention is that these new complexes of nucleic acid and substituted polymer are, in the presence of serum, just as effective as (or even more effective than) in the absence of serum for transfection of cells.

In one of its most general definitions, the invention relates to a complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, and/or of endosomes in a weakly acid medium,

- the abovementioned residues also having the following properties:

  . they carry a functional group which enables them to be bonded to the abovementioned polymer,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

  . they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

  . they carry at least one hydroxyl group,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), in particular on the hydroxyl groups of the abovementioned residues, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

Destabilization of membranes is understood as meaning a modification of the membrane which leads either to an increase in its permeability with respect to low molecular weight and possibly high molecular weight molecules of the solution (including nucleic acids, plasmids or complexes), or fusion with another membrane.

The membrane permeability can be measured by fluorescence microscopy in the following manner:

Adhesive cells are incubated at 37°C for 15 to 30 minutes with 0.5 ml DMEM culture medium with serum containing 5 mg/ml dextran (Mw 4,000) labelled with fluorescein isothiocyanate (FTC-dextran) and a DNA/histidylated polylysine complex. The cells are then washed and incubated at 37°C for 15 to 30 minutes with culture medium comprising 10% foetal bovine serum. The cells are then fixed for 5 minutes in a saline phosphate buffer solution comprising 4% *p*-formaldehyde and the fluorescence is analysed with a confocal fluorescence microscope (MRC600 BioRad). In the absence of membrane permeabilization, the fluorescence originating from FTC-dextran is located exclusively in the vesicles. In the presence of a membrane permeabilization agent, the fluorescence originating from the FTC-dextran is also observed in a diffuse manner in the cytosol and the nucleus of the cells.

The fusion of membranes in the presence of DNA/histidylated polylysine complexes is measured easily in model systems, such as liposomes, using a lipid mixture method such as that described in Struck D.K. *et al.* (Use of resonance energy transfer to monitor membrane

fusion. (1981) Biochemistry 20:4093-4099). Briefly, liposomes made up of dioleoyl-phosphatidylcholine (DPOC), into the membrane of which are inserted N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and octadecylrhodamine (R18) as a fluorescence marker, and liposomes without fluorescence markers are used. The fluorescence of NBD (λex = 470 nm; λem = 530 nm) is measured in the absence and presence of DNA/histidylated polylysine complexes at various pH values. The fusion of the liposomes induces a reduction in the fluorescence of NBD as a consequence of a reduction in the transfer energy between rhodamine and NBD.

These new complexes of nucleic acid and substituted polymer containing weakly basic groupings which can be protonated (cation) in a slightly acid medium in the presence of serum are more suitable for gene transfer *in vivo* than the DNA/polylysine or DNA/gluconylated polylysine complexes which are active only in the presence of auxiliaries such as chloroquine or fusiogenic and/or permeabilizing peptides.

The residues cause destabilization of cell membranes due to their property of being protonatable in an acid medium.

The residues which cause destabilization of cell membranes are proton captors which limit acidification of endosomes and, as a consequence, hinder fusion between late endosomes and liposomes. It should be noted that lysosomes are vesicles containing a large number of hydrolases and that these lysosomes are very effective in degrading biological macromolecules in general and nucleic acids in particular.

The term "weakly acid medium" means a medium of which the pH is less than that of plasma or serum, for example a pH of less than 7.4.

The term according to which the residues "are not active with respect to the recognition signal recognized by a cell membrane receptor" indicates that, on the one hand, to date there are no known receptors which are specific to these residues and, on the other hand, that these residues are not used as ligands.

A molecule or a molecular complex is active with respect to a recognition signal if it can be recognized selectively by a receptor, that is to say it plays the role of a ligand, an agonist or an antagonist.

Recognition signal recognized by a cell membrane receptor generally means a ligand (molecule or molecular complex) which is capable of being recognized selectively by the said receptor (ligand-receptor affinity $\geq 10^3$ l/mole).

The invention particularly relates to a complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, and/or of endosomes in a weakly acid medium,

- the abovementioned residues also having the following properties:

  . they are bases of which the pK in an aqueous medium is less than 8, such that a proportion of greater than 50% of these bases bonded to a cationic polymer is not protonated in a neutral medium of pH 7.4,

  . they carry a functional group which enables them to be bonded to the abovementioned polymer,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

  . they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

  . they carry at least one hydroxyl group,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

  . by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

  . on some of the abovementioned non-charged residues causing a reduction in the

charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

The invention also relates to a complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, in a weakly acid medium,

- the abovementioned residues also having the following properties:

. they belong to the family of compounds which carry an imidazole nucleus,

. they belong to the family of quinolines,

. they belong to the family of pterines,

. they belong to the family of pyridines,

. the abovementioned residues carry a functional group which enables them to be bonded to the abovementioned polymer,

. they can carry at least one free NH_3^+ function,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate,

facilitating salting out of the nucleic acid in the course of dissociation of the complex, with the proviso that all the abovementioned residues contain at least 30% of free NH_3^+ functions,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

. they carry at least one hydroxyl group,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

The recognition signals can also cause a reduction in the positive charges of the polymeric conjugate if they are themselves neutral or acid and they are bonded to the polymer by substitution of an NH_3^+ function causing the loss of the + charge.

The recognition signals are molecules of low molecular weight (< 5,000 daltons).

The number of molecules of the recognition signal bonded to the modified polymer can be:

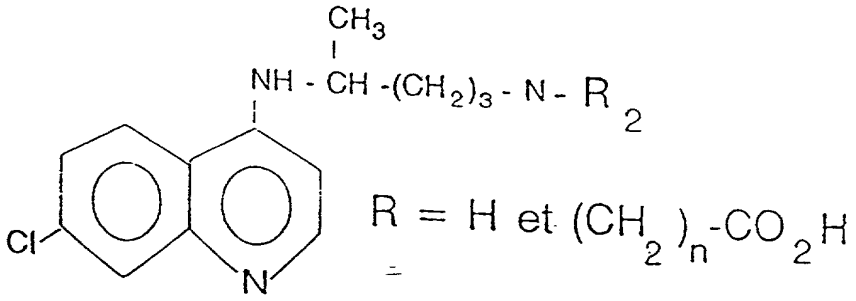
- for a signal molecule of very high affinity with respect to its receptor (Ka > 10^7 l/mole), about 0.5 to 5, advantageously 1 molecule for about 10,000 monomer units of the

substituted polymer, that is to say 1 molecule for about 50 molecules of the substituted polymer;

- for a signal molecule of high affinity with respect to its receptor (Ka between 10^5 l/mole and 10^7 l/mole), about 0.5 to about 10, advantageously 1 molecule for about 200 monomer units of the substituted polymer, that is to say 1 molecule for about 1 molecule of substituted polymer;

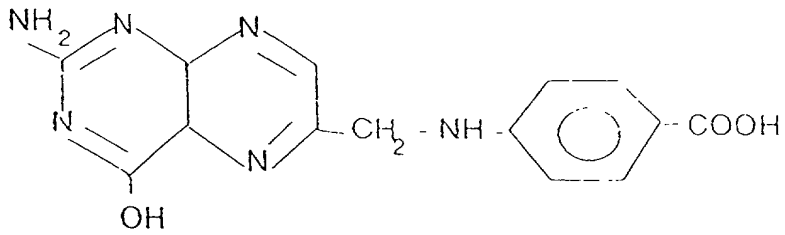
- for a signal molecule of moderate affinity with respect to its receptor (Ka < 10^5 l/mole), about 10 to about 100, advantageously 50 molecules for about 200 monomer units of the substituted polymer, that is to say 50 molecules for about 1 molecule of substituted polymer.

The family of quinolines is represented by the following formula:

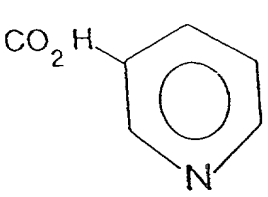


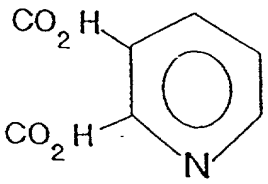
in which n has a value from 1 to 10, preferably 1 to 3.

The family of pterines is represented by the following formula:



The family of pyridines is represented by the following formulae:

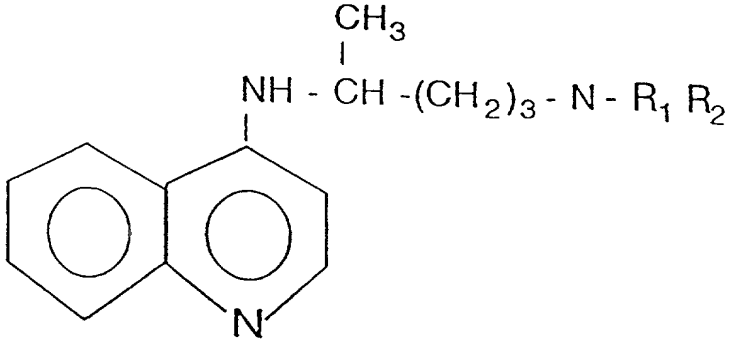




The invention also relates to a complex in which the residues causing destabilization of cell membranes in a weakly acid medium are

- alkylimidazoles in which the alkyl radical contains 1 to 10, in particular 2 to 6 carbon atoms, and in which only one of the nitrogen atoms of the imidazole nucleus is substituted,

- or quinolines of the formula:



in which R_1 represents H and R_2 represents $(CH_2)_n$-CO_2-H, n being an integer varying from 1 to 10, and preferably having a value of 1 to 3.

The invention also relates to a complex in which the residues causing destabilization of cell membranes are chosen from: histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(3-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-

4-yl)ethylamine, β-alanyl-histidine-(carnosine), 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid,

in which

- the optional free NH_3^+ function of the abovementioned residues (for example histidine) can also be substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

The invention relates to a complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, in particular residues of lysine or ornithine, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, by residues causing a destabilization of cell membranes in a weakly acid medium,

- the abovementioned residues also having the following properties:

  . they carry an imidazole nucleus,

  . they can carry at least one free NH_3^+ function,

  . they are not active with respect to the recognition signal,

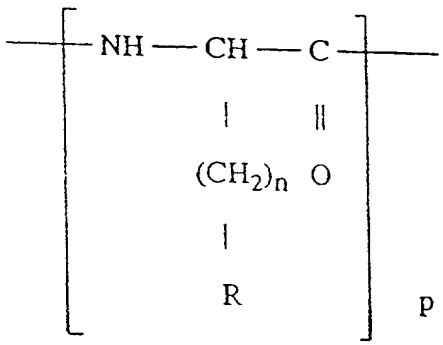
- the remaining free NH_3^+ functions of the abovementioned monomer units also being substituted to the extent of about 1% to about 60% by a molecule which constitutes a recognition signal recognized by a cell membrane receptor, this recognition signal having a molecular weight of less than 5,000, and it being possible for this recognition signal to be present in an amount of one molecule for about 200 units of polymeric conjugate or about 60 molecules for about 200 units of polymeric conjugate,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

The term "not active with respect to the recognition signal" indicates that on the one hand to date there are no known receptors which are specific to these residues and, on the

other hand, these residues are not used as ligands.

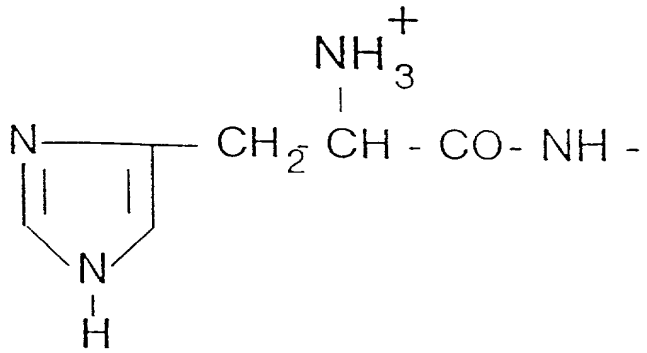
The invention also relates to a complex in which the polymer contains a polymeric grouping of the following formula (I):



(I)

in which:

- p is an integer varying from 15 to 900, preferably 100 to 300,
- n is an integer varying from 1 to 6, and preferably has the value 4,
- this polymeric grouping contains radicals R among which:
  . 10% to 45% of the number of radicals R representing a residue carrying an imidazole nucleus and optionally a free NH_3^+ function, in particular a histidyl residue, it being possible for R to be represented by the formula:



it being possible for the optional NH_3^+ function of the abovementioned residues also to be substituted by a molecule which constitutes a recognition signal,

. 10% to 90% of the number of radicals R representing free ϖ-amino NH_3^+ and optionally being substituted to the extent of 0 to 50% by a molecule which constitutes a recognition signal, in particular to the extent of 0 to 60, advantageously 1 molecule for about 200 units, or to the extent of 2 to 100, advantageously 50 molecules for about 200 units, and/or

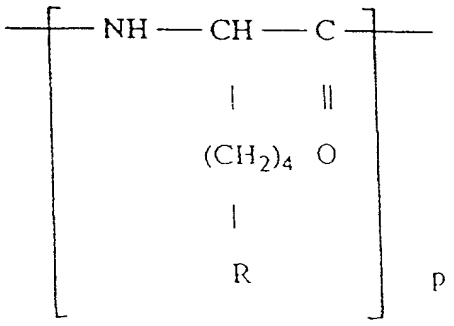
. it also being possible for R to be made up to the extent of 0 to 45% of a group $NH\text{-}CO\text{-}(CHOH)_m\text{-}R_1$, in particular a dihydroxypropionylamido, erythronylamido, threonylamido, ribonylamido, arabinylamido, xylonylamido, lyxonylamido, gluconylamido, galactonylamido, mannonylamido, glycoheptonylamido or glycooctonylamido radical, m is an integer from 2 to 15, preferably 2 to 7, R_1 represents H or an alkyl radical having 1 to 15 carbon atoms, in particular CH_3, it being possible for these radicals to be substituted by a molecule which constitutes a recognition signal, with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

In this class of complexes of the invention, the polymer is polylysine or polyornithine.

As shown by the examples, HepG2 (human hepatocarcinoma) cells are transfected effectively by polylysine substituted by 70 histidyl residues.

Polylysine substituted by 30 ± 10% histidine has allowed transfection of various cells (human and murine) with a high efficiency, modulated according to the cell type and the promoter used.

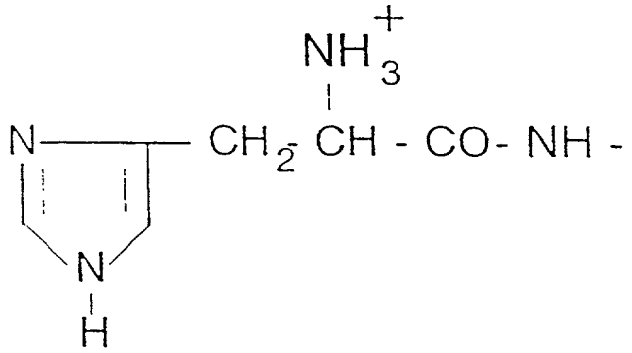
The invention also relates to a complex in which the polymer comprises a polymeric grouping of the following formula (II):



in which:

- p has the meanings indicated above,

- 10% to 45% of the number of radicals R represent a residue carrying an imidazole nucleus and optionally a free NH_3^+ function, in particular a histidyl residue, it being possible for R to be represented by the formula



it being possible for the NH_3^+ functions of the abovementioned residues also to be substituted by a molecule which constitutes a recognition signal,

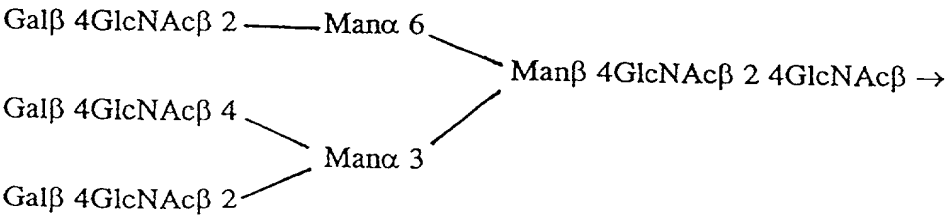
- the remainder of the radicals, that is to say 30% to 90% of the number of radicals R, representing ϖ-amino NH_3^+, and it being possible for 0 to 45% of the radicals R to be substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

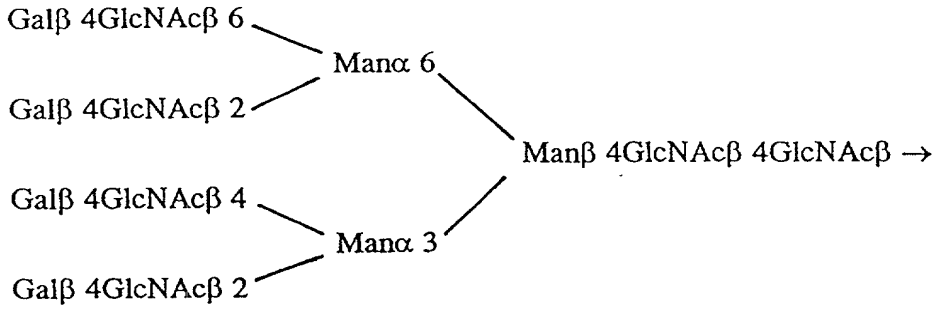
The invention also relates to a complex, which is characterized in that the recognition signal is chosen from:

A) - simple or complex osides recognized by membrane lectins and chosen from:

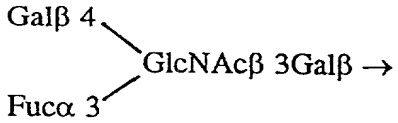
a. Asialo-oligoside of the type of triantennar lactosamine: asialoglycoprotein receptor



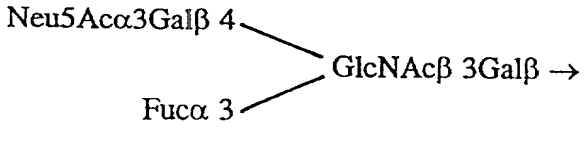
b. Asialo-oligoside of the type of tetraantennar lactosamine: asialoglycoprotein receptor



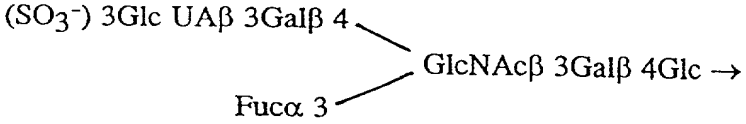
c. Lewis x: LECAM 2/3



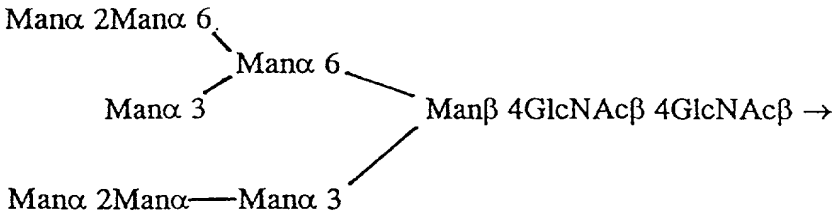
d. Lewis x sialyl: LECAM 3/2



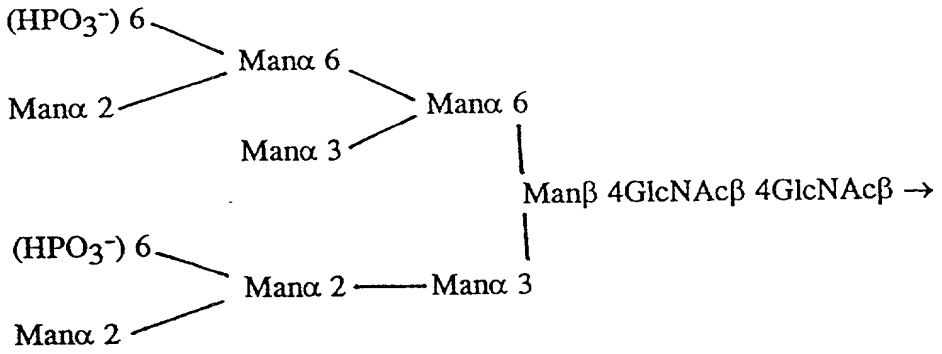
e. Sulphated Lewis x derivative (HNK1): LECAM 1



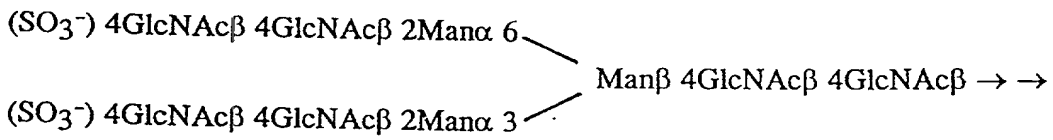
f. Oligomannoside: mannose receptor



g. Phosphorylated oligomannoside: mannose 6-phosphate receptor



h. Oligosaccharide of the type of sulphated lactosamine: sulphated GalNAc 4 receptor



B) Peptides

a. anti-inflammatory peptides or certain of their fragments recognized by receptors of the vascular wall, such as

- vasodilator intestinal polypeptide (VIP)

HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2

- atrial natriuretic polypeptide (ANP)

SLRRSSCFGGRMDRIGAQSGLGCNSFRY

- lipocortin

HDMNKVLDL

- bradykinin

RPPGFSPFR;

b. ligand peptides of integrins, such as peptides containing the sequence RGD, fibronectin ligand;

c. chemiotactic factors, such as formyl-peptides and their antagonists: FMLP, (N-formyl-Met-Leu-Phe);

d. peptide hormones, such as

α-MSH: Ac-SYSMEHFRWGKPV-NH_2 and their antagonists.

C) Natural metabolites, such as:

- biotin,
- carnitine,
- tetrahydrofolate and folic acid, which can be both a recognition signal with respect to certain cells having suitable receptors and a destabilizer of cell membranes.

The invention also relates to a complex, which is characterized in that the nucleic acid can be chosen from:

a) marker genes, such as

- genes containing luciferase,
- green protein of the jellyfish *Aequarea victoria*,
- genes containing β-galactosidase,
- genes containing chloramphenicol acetyltransferase,
- genes which confer resistance to an antibiotic, such as hygromycin, neomycin etc....;

b) genes with a therapeutic purpose, such as

- receptors of lipoproteins of low-density, which are deficient in cases of hypercholesterolaemia,
- coagulation factors: factors VIII and IX,
- phenylalanine hydroxylase (phenylketonuria),
- adenosine deaminase (ADA immunodeficiency),
- lysosomal enzymes, such as β-glucosidase in the case of Gaucher's disease,
- dystrophin and minidistriphin (myopathy),
- tyrosine hydroxylase (Parkinson),
- neurone growth factors (Alzheimer),
- CFTR cystic fibrosis transmembrane conductance regulator (cystic fibrosis),
- alpha-1-antitrypsin,
- cytokines (interleukins, TNF tumour necrosing factor),
- thymidine kinase of the Herpes simplex virus,
- proteins of MHC, major histocompatibility complex, in particular HLA-B7,
- cytosine deaminase,
- genes which code for sense and antisense RNAs,
- genes which code for ribozymes,

c) genes for the purpose of vaccines

- genes which code for viral antigens (vaccination), for example: the gene which codes for the nucleoprotein of the influenza virus.

The invention also relates to a complex in which:

- the polymer, in particular polylysine, has a degree of polymerization of about 15 to about 900, preferably 200,

- the free NH_3^+ functions of the lysine units being substituted in a ratio of 35% by histidyl residues and optionally by a molecule which constitutes a recognition signal for 1 to 50 residues of lysine, where the said signal molecule has an affinity of at least 10^5 l $mole^{-1}$ with respect to the receptor of the cell which the complex is to target, or optionally by 20 to 100 molecules of recognition signal for 200 lysine residues, where the said signal molecule has an affinity of less than 10^5 l $mole^{-1}$ with respect to the said receptor,

- the nucleic acid has a molecular weight of about 10^6 to about 10^8, in particular 3.10^6 to 30.10^6,

- the ratio between the average number of base pairs of the nucleic acid per molecule of monomer unit, in particular lysine, is about 0.2 to about 6, preferably about 0.4 to about 0.6.

With regard to the affinities:

- for a signal molecule of very high affinity with respect to its receptor (Ka > 10^7 l/mole), about 0.5 to 5, advantageously 1 molecule for about 10,000 monomer units of the substituted polymer, that is to say 1 molecule for about 50 molecules of substituted polymer;

- for a signal molecule of high affinity with respect to its receptor (Ka between 10^5 l/mole and 10^7 l/mole), about 0.5 to about 10, advantageously 1 molecule for about 200 monomer units of the substituted polymer, that is to say 1 molecule for about 1 molecule of substituted polymer;

- for a signal molecule of moderate affinity with respect to its receptor (Ka < 10^5 l/mole), about 10 to about 100, advantageously 50 molecules for about 200 monomer units of the substituted polymer, that is to say 50 molecules for about 1 molecule of substituted polymer.

The invention also relates to a positively charged polymeric conjugate containing units carrying free NH_3^+ functions and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, in a weakly acid medium,

- the abovementioned residues also having the following properties:

  . they carry a functional group which enables them to be bonded to the abovementioned polymer,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

  . they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

. they carry at least one hydroxyl group,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

. it being possible for the hydroxyl groups of the abovementioned non-charged residues to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysines), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

The invention also relates to a polymeric conjugate as defined above or containing a polymeric grouping as defined above.

According to an advantageous embodiment of the invention, the polymeric conjugate is chosen from histidylated polylysine substituted by lactose, histidylated polylysine substituted by a complex oligoside such as Lewis^b, histidylated polylysine substituted by the peptide ANP, or histidylated polylysine substituted by biotin.

The polymeric conjugates of the invention can be prepared by one of the methods described in the following tables:

Table I: Methods for preparation of polymeric conjugates with recognition signals bonded to certain monomeric units of the polymer.

The respective order of introduction of residues responsible for destabilization, those responsible for the reduction in charge and recognition signals on to the polymer has been

indicated by 1, 2 and 3.

Method	POLYMER residues responsible for destabilization	POLYMER residues responsible for the reduction in charge	recognition signal
I	1	-	2
II	2	-	1
III	1	2	3
IV	1	3	2
V	2	1	3
VI	3	1	2
VII	2	3	1
VIII	3	2	1

Table II: Methods for preparation of polymeric conjugates with recognition signals bonded to certain residues responsible for destabilization of membranes.

The respective order of introduction of residues responsible for destabilization, those responsible for the reduction in charge and recognition signals on to the polymer has been indicated by 1, 2 and 3.

Method	POLYMER residues responsible for destabilization	POLYMER residues responsible for the reduction in charge	recognition signal
IX	1	-	2
X	1	2	3
XI	1	3	2
XII	2	1	3

Table III: Methods for preparation of polymeric conjugates with recognition signals bonded to certain residues responsible for the reduction in charge.

The respective order of introduction of residues responsible for destabilization, those responsible for the reduction in charge and recognition signals on to the polymer has been indicated by 1, 2 and 3.

	POLYMER residues		
Method	responsible for destabilization	responsible for the reduction in charge	recognition signal
XIII	2	1	3
XIV	3	1	2
XV	1	2	3

The residues responsible for destabilization of membranes are chosen from: histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(1-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)-ethylamine, 2-(3-methyl-imidazol-4-yl)-ethylamine, β-alanyl-histidine, 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxy-quinoline, N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid.

The residues responsible for the reduction in charge are chosen from: dihydroxypropionyl, erythronyl, threonyl, ribonyl, arabinyl, xylonyl, lyxonyl, gluconyl, galactonyl, mannonyl, glycoheptonyl, glycooctonyl.

The recognition signals are chosen from: osides, oligosides, peptides, metabolites, agonists and antagonists.

By way of example, the various methods of the tables are described:

I) The recognition signals are bonded to certain monomeric units of the polymer after introduction of the residues causing a destabilization of the cell membrane in the following manner:

A) Method I

Nicotinylated polylysine

a) Monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing destabilization of cell membranes. For example, polylysine (in particular in the *p*-toluenesulphonate form) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause destabilization of cell membranes (in particular nicotinic acid) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

b) The recognition signals are bonded to certain ε-amino groupings of lysyl residues of the polymer.

By way of example of the bonding of recognition signals on to nicotinylated polylysine, the bonding of osides or oligosides is indicated below.

1) Bonding of osides .

Simple osides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993 21: 871-878).

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1, 269-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993, 21: 871-878).

Histidylated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues carrying a function which allows subsequent bonding of other molecules, such as those which will constitute a recognition signal, for example, after reaction in an organic medium with an N-hydroxysuccinimide ester of dithiopyridine-propionic acid or its derivatives.

b) The monomer units of the polymer carrying a free NH_3^+ function are then partly substituted by residues causing a destabilization of cell membranes, for example, after reaction in an organic medium with histidine, in which the αNH_3^+ group and the NH group of the imidazole nucleus are protected by tert-butyloxycarbonyl, in the presence of a coupling agent, such as benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate. After reaction and purification, the amino functions of the histidyl residues of the polymer obtained are deprotected.

c) The recognition signals are bonded to dithiopyridyl groupings of the polymer.

By way of example of the bonding of recognition signals on to histidylated polylysine, the bonding of osides or oligosides is indicated below.

1) Bonding of osides.

Simple osides derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275) are reduced and bonded in an aqueous medium, buffered at a neutral pH, to dithiopyridyl functions of the polymer.

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275) are reduced and bonded in an aqueous medium, buffered at a neutral pH, to dithiopyridyl functions of the polymer.

B) Method III

Nicotinylated and gluconylated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing destabilization of cell membranes. For example, gluconylated polylysine is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause destabilization of cell membranes (in particular nicotinic acid) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

b) The monomer units of the polymer carrying an NH_3^+ function which is still free are then partly substituted by non-charged residues causing a reduction in charge. As regards bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and an activated hydroxylated organic acid (in particular δ-gluconolactone).

c) The recognition signals are bonded to certain ε-amino groupings of the lysyl residues of the polymer.

By way of example of the bonding of recognition signals on to histidylated polylysine, the bonding of osides or oligosides is indicated below.

1) Bonding of osides .

Simple osides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993 21: 871-878).

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.* (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates. Carbohyd. Letters 1:69-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993, 21:871-878).

C) Method V

Gluconylated and histidylated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues carrying a function which allows subsequent bonding of other molecules. As regards the bonding of recognition signals, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and the N-hydroxysuccinimide ester of dithiopyridine-propionic acid or its derivatives.

b) The monomer units of the polymer carrying an NH_3^+ function which is still free are then partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and an activated hydroxylated organic acid (in particular δ-gluconolactone).

c) The monomer units of the polymer carrying an NH_3^+ function which is still free are then partly substituted by residues causing a destabilization of cell membranes. As regards the bonding of residues causing a destabilization of cell membranes, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular histidine in which the αNH_3^+ group and the NH group of the imidazole nucleus are protected by tert-butyloxycarbonyl) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate). After purification, the protected amino functions of the histidyl residues are deprotected.

d) The recognition signals are bonded to dithiopyridyl groupings of the polymer.

By way of example of the bonding of recognition signals on to histidylated polylysine, the bonding of osides or oligosides is indicated below.

1) Bonding of osides .

Simple osides derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New

synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275) are reduced and bonded in an aqueous medium, buffered at a neutral pH, to dithiopyridyl functions of the polymer.

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275) are reduced and bonded in an aqueous medium, buffered at a neutral pH, to dithiopyridyl functions of the polymer.

Gluconylated and nicotinylated polylysine.

a) The monomer units of the polymer carrying an NH_3^+ function which is still free are partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and an activated hydroxylated organic acid (in particular δ-gluconolactone).

b) The monomer units of the polymer carrying a free NH_3^+ function are then partly substituted by residues causing a destabilization of cell membranes. For example, gluconylated polylysine is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular nicotinic acid) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

c) The recognition signals are bonded to certain ε-amino groupings of the lysyl residues of the polymer.

By way of example of the bonding of recognition signals on to histidylated polylysine, the bonding of osides or oligosides is indicated below.

1) Bonding of osides .

Simple osides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*,

(Nucleic Acids Res. 1993, 21:871-878).

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993, 21: 871-878).

II) The recognition signals are bonded to certain monomeric units of the polymer before introduction of residues causing a destabilization of cell membranes, in the following manner:

A) Method II

Nicotinylated polylysine

These substitutions follow any one of the protocols known to the person skilled in the art.

a) By way of example of the bonding of recognition signals on to polylysine (in particular in the form of the *p*-toluenesulphonate) dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), the bonding of osides and oligosides is indicated below.

1) Bonding of osides .

Simple osides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993 21: 871-878).

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C.

and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1, 269-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993, 21: 871-878).

b) As regards the bonding of residues which cause destabilization of membranes, for example, polylysine substituted by osides or oligosides is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular nicotinic acid) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

B) Method VI

Gluconylated and nicotinylated polylysine

These substitutions follow any one of the protocols known to the person skilled in the art.

a) The monomer units of the polymer carrying an NH_3^+ function which is still free are partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and an activated hydroxylated organic acid (in particular δ-gluconolactone).

b) By way of example of the bonding of recognition signals on to the gluconylated polylysine dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), the bonding of osides or oligosides is indicated below.

1) Bonding of osides .

Simple osides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993 21: 871-878).

2) Bonding of oligosides

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are

obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain ε-amino functions of free lysyl residues of polylysine as described previously in Midoux *et al.*, (Nucleic Acids Res. 1993, 21: 871-878).

c) As regards the bonding of residues which cause destabilization of membranes, for example, polylysine substituted by osides or oligosides is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecule which cause a destabilization of cell membranes (in particular nicotinic acid) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

III) The recognition signals are bonded to certain destabilizing residues

A) Method IX

Histidylated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing a destabilization of cell membranes. For example, after reaction in an organic medium with histidine, in which the NH_3^+ group and the NH group of the imidazole nucleus are protected by tert-butyloxycarbonyl, in the presence of a coupling agent, such as benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate. After reaction and purification, the amino functions of the histidyl residues of the polymer obtained are deprotected.

b) The recognition signals are bonded to certain NH_3^+ groupings of the residues causing destabilization of membranes.

By way of example of the bonding of recognition signals on to the histidylated polylysine, the bonding of oligosides is indicated below.

Complex oligosides, such as the asialo-oligosides of the triantennar or tetraantennar or Lewis type, are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation

et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275). The glycopeptides in the form of phenyl isothiocyanate derivatives are bonded in an aqueous medium, buffered at a neutral pH, to certain NH_2 functions of the histidyl residues. At this pH, bonding to the lysine NH_3^+ is very weak, or even impossible.

B) Method XII

Gluconylated and histidylated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and an activated hydroxylated organic acid (in particular δ-gluconolactone).

b) the monomer units of the polymer carrying an NH_3^+ function which is still free are then partly substituted by residues causing a destabilization of cell membranes. As regards the bonding of residues causing a destabilization of cell membranes, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular histidine, in which the αNH_3^+ group and the NH group of the imidazole nucleus are protected by tert-butyloxycarbonyl) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate). After purification, the protected amino functions of the histidyl residues are deprotected.

c) The recognition signals are bonded to certain NH_3^+ groupings of residues causing a destabilization of membranes.

By way of example of the bonding of recognition signals on to histidylated polylysine, the bonding of oligosides is indicated below.

Complex oligosides, such as bi-, tri- or tetra-antennar or Lewis asialo-oligosides are obtained in the form of phenyl isothiocyanate derivatives of glycopeptides by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New oligoside derivatives, a process for their preparation and their uses]) and

Sdiqui *et al.* (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275). Glycopeptides in the form of phenyl isothiocyanate derivatives are bonded to certain αNH_2 functions of histidyl residues; at this pH, bonding to the lysine NH_3^+ is very weak or even impossible.

C) Method IX

Imidazolated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing a destabilization of cell membranes. As regards the bonding of residues causing a destabilization of cell membranes, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular 4-carboxymethyl-imidazole) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

b) The recognition signals are bonded to certain imidazole nuclei of the residues causing a destabilization of membranes.

By way of example of the bonding of recognition signals to the imidazolated polylysine, the bonding of peptides or glycopeptides is indicated below.

The imidazole residues can easily be alkylated in a neutral medium by compounds which have an activated grouping, such as, for example, iodoacetamide or its derivatives; this is known from the works by Korman S. and Clarke H.T. in 1956 (J. Biol. Chem. 113:133). The alkylation of imidazoles by an iodoacetamide derivative is carried out at a pH close to neutrality, for example 7.0. At this pH, the ε-amino groups of the lysine are not affected, but they would be at a much more alkaline pH of 9 or beyond. The recognition signals of a peptide or glycopeptide nature (Monsigny *et al.*, French Patent 9407738, 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses] and Sdiqui *et al.*, 1995 New synthesis of glyco-amino acid conjugates. Carbohyd. Letters 1:269-275) can easily be substituted by an iodoacetamide grouping. The derivatives of the type ICH_2CONHR : I-CH_2-CO-NH-peptide or I-CH_2-CO-NH-glycopeptide, are bonded in an aqueous medium, buffered at a neutral pH, to the 3-nitrogen, and with a lower efficiency to the 1-nitrogen, leading to stable *N*-carboxymethyl derivatives. It is also possible to use bromoacetamide derivatives,

which are also excellent reagents for alkylation of imidazole residues (see, for example, Henrikson *et al.*, 1965 J. Biol. Chem. 240:2921). This type of substitution, with the proviso of substitution of a small number of imidazole residues by recognition signals having a high affinity sufficient for their receptor, does not cause the polymer substituted by imidazole residues to lose its capacity for destabilizing membranes at a slightly acid pH.

IV) The recognition signals are bonded to certain neutralizing residues.

A) Method XIII

Gluconylated and imidazolated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and two activated hydroxylated organic acids (in particular 6-deoxy-6-iodo-δ-gluconolactone in one part and δ-gluconolactone in 10 to 50 parts).

b) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing a destabilization of cell membranes. As regards the bonding of residues causing a destabilization of cell membranes, for example, a polylysine salt (in particular in the form of the *p*-toluenesulphonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular 4-carboxymethyl-imidazole) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

c) The recognition signals are bonded to certain neutralizing residues.

By way of example of the bonding of recognition signals on to the gluconylated and histidylated polylysine, the bonding of oligosides is indicated below.

Complex oligosides, such as the bi-, tri- or tetra-antennar or Lewis asialo-oligosides derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation at leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid

conjugates, Carbohyd. Letters 1:269-275) are reduced by triscarboxyethylphosphine in a neutral medium (pH about 7.0), for example, and bonded in an aqueous medium, buffered at a slightly alkaline pH (at about pH 8.5), to the 6-deoxy-6-iodo-gluconyl residues of the polymer. This type of substitution, with the proviso of substitution of a small number of imidazole residues by recognition signals having a high affinity for their receptor, does not cause the polymer substituted by imidazole residues to lose its capacity for destabilization of membranes at a slightly acid pH.

B) Method XIII

Gluconylated and imidazolated polylysine

a) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by non-charged residues causing a reduction in charge. As regards the bonding of residues causing the reduction in charge, for example, a polylysine salt (in particular in the form of the *p*-toluenesulfonate is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine) and two activated hydroxylated organic acids (in particular 6-bromoacetamido-L-gulono-1,5-lactone in one part and δ-gluconolactone in 10 to 50 parts). 6-bromoacetamido-L-gulono-1,5-lactone is obtained after reduction by cyanoborohydride of the imine obtained by mixing an ammoniacal solution (NH_4OH or $(NH_4)_2CO_3$) and a solution of a uronic acid, for example glucuronic acid, and then by acylation of the amine by an activated bromoacetate, for example bromoacetic anhydride or succinimidyl bromoacetate.

b) The monomer units of the polymer carrying a free NH_3^+ function are partly substituted by residues causing a destabilization of cell membranes. As regards the bonding of residues causing a destabilization of cell membranes, for example, a polylysine salt (in particular in the form of the *p*-toluenesulfonate) is dissolved in an organic solvent (in particular dimethylsulphoxide) in the presence of a base (in particular diisopropylethylamine), molecules which cause a destabilization of cell membranes (in particular 4-carboxymethyl-imidazole) and a coupling agent (in particular benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate).

c) The recognition signals are bonded to certain neutralizing residues.

By way of example of the bonding of recognition signals on to the gluconylated and histidylated polylysine, the bonding of oligosides is indicated below.

Complex oligosides, such as the bi-, tri- or tetra-antennar or Lewis asialo-oligosides

derived from glycopeptides with a dithiopyridyl function (*pyro*glutamyl-NH-$(CH_2)_2$-S-S-pyridine) by a method described in Monsigny *et al.*, (French Patent 9407738, Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation at leurs applications [New derivatives of oligosides, a process for their preparation and their uses]) and Sdiqui *et al.*, (1995 New synthesis of glyco-amino acid conjugates, Carbohyd. Letters 1:269-275) are reduced by triscarboxyethylphosphine in a neutral medium (pH about 7.0), for example, and bonded in an aqueous medium, buffered at a slightly alkaline pH (at about pH 8.5), to the bromoacetamido-gluconyl residues of the polymer. This type of substitution, with the proviso of substitution of a small number of imidazole residues by recognition signals having a high affinity for their receptor, does not cause the polymer substituted by imidazole residues to lose its capacity for destabilization of membranes at a slightly acid pH.

The nucleic acid/polymeric conjugate complex is obtained by mixing a solution of the nucleic acid in question and a solution of the polymeric conjugate. The said solutions are preferably prepared from physiological serum or a buffer or a cytocompatible medium.

According to an advantageous embodiment of the invention, a complex as described above or a conjugate as described above is used for the *in vitro, ex vivo* or *in vivo* transfection of cells with the aid of a gene, in particular those defined previously.

According to an advantageous embodiment of the invention, a complex or a conjugate as described above is used, being characterized in that the cells are chosen from;

- cells of haematopoietic strains;
- dendritic cells;
- liver cells;
- skeletal muscle cells;
- skin cells:
  - . fibroblasts,
  - . keratinocytes,
  - . dendritic cells,
  - . melanocytes;
- cells of the vascular walls;
  - . endothelial;
  - . smooth muscle;

- epithelial cells of the respiratory tract;
- cells of the central nervous system;
- cancerous cells;
- cells of the immune system, such as lymphocytes, macrophages, NK cells etc.

According to an advantageous embodiment of the invention, the method of *in vitro* or *ex vivo* transfection is characterized in that a complex as described previously is brought into contact with a medium containing cells to be transfected under conditions such that there is:

- passage of the complex from the medium into the cytoplasm of the cells,
- salting out of the nucleic acid involved in the abovementioned complex in the cytosol and/or the nucleus of the cells,
- transcription and expression of the nucleic acid in the transfected cells,
- expression of the protein corresponding to the transfected gene.

The invention also relates to a pharmaceutical composition, which is characterized in that it comprises, as the active substance, at least one of the complexes as described above, or at least one of the conjugates as described above, in combination with a pharmaceutically acceptable vehicle.

According to an advantageous embodiment of the invention, a complex as described above or a conjugate as described above is used for the preparation of a medicament intended, for example, for treatment of congenital or acquired metabolic deficiency, or treatment of tumours, or for the preparation of a vaccine, for example a vaccine against influenza.

The invention also relates to a set or kit comprising:

- a polymeric conjugate as described above, such as polylysine substituted by a residue causing a destabilization of cell membranes in a weakly acid medium, this polymeric conjugate being capable of optionally carrying a recognition signal, which is or is not bonded beforehand to the abovementioned polymeric conjugate, the said recognition signal being a function of the cell to be targeted,
- optionally a plasmid containing at least one gene to be transferred, and optionally the system for regulation of the expression of the abovementioned gene,
- reagents which allow optional bonding of the recognition signal on to the abovementioned polymeric conjugate,
- reagents which allow the formation of a complex as described above, or between the

polymeric conjugate and the gene to be transferred, or between the polymeric conjugate and a plasmid containing the gene to be transferred,

- reagents which allow transfection of the cell by the abovementioned complex.

DESCRIPTION OF THE FIGURES:

Figure 1

This shows a fragment of polylysine (DP 190) partly substituted by histidyl residues.

Figure 2

This shows the NMR spectrum at 300 MHz in D_2O of polylysine (DP 190) substituted by 70 histidyl residues:

1.28 to 1.88 ppm: 6 protons of carbons 3, 4 and 5 of substituted or unsubstituted lysines.

2.39 ppm: protons of the CH_3 group of *p*-toluenesulphonate

2.75 ppm: DMSO plot

2.99 ppm: 2 protons of carbon 6 of an unsubstituted lysyl residue

3.15 ppm: 2 protons of carbon 6 of a substituted lysyl residue

3.35 ppm: 2 protons of carbon 9 of a histidyl residue

4.36 ppm: 2 protons of carbons 2 and 8

4.78 ppm: peak of water

7.36 ppm: 2 protons (doublet, ortho-coupling constant = 7.97 Hz) of the protons of carbons 2 and 6 of the aromatic ring of *p*-toluenesulphonate

7.42 ppm: 1 proton of carbon 11 of a histidyl residue

7.71 ppm: 2 protons (doublet, ortho-coupling constant = 8.01 Hz) of the protons of carbons 3 and 5 of the aromatic ring of *p*-toluenesulphonate

8.7 ppm: 1 proton of carbon 12 of a histidyl residue.

Figure 3

This relates to the preparation of polylysine (DP 190) partly substituted by 70 histidyl residues.

Poly-L-lysine in the hydrobromide form (average molecular weight 40,000; average

degree of polymerization 190) (1 g in 200 ml H_2O) originating from Bachem Feinchemikalien (Budendorf, Switzerland) is first passed over an anion exchange column (Dowex 2 x 8, OH^- form; 35 x 2.5 cm) in order to remove the bromide, which is toxic to cells. The polylysine solution is neutralized with a 10% solution of *p*-toluenesulphonic acid in water and then lyophilized.

The polylysine is partly substituted with histidyl residues as follows: the polylysine in the form of the *p*-toluenesulphonate (50 mg; 0.96 µmole), dissolved in 3 ml DMSO (dimethylsulphoxide) in the presence of diisopropylethylamine (42 µl; 288 µmoles) is reacted for 24 hours at 20°C with 32 mg (Boc)His(Boc)-OH (96 µmoles) in the presence of 43 mg benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (97 µmoles). The histidyl residues are then deprotected in the presence of 20 ml of a mixture of water and trifluoroacetic acid (TFA) (50/50 V/V) for 24 hours at 20°C. The water and the TFA are removed by evaporation under reduced pressure. The polymer is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and collected after renewed centrifugation. The residue is taken up in distilled water and the solution is lyophilized. The number x of histidyl residues bonded per molecule of polylysine is determined by proton NMR as follows:

$$x = 6(h_{8.7}/h_{Lys})DP$$

where $h_{8,7}$ is the integral of the peak at 8.7 ppm corresponding to the proton of carbon 12 of a histidyl residue, h_{Lys} is the integral of peaks between 1.28 and 1.88 ppm corresponding to 6 protons of carbons 3, 4 and 5 of lysine residues and DP is the degree of polymerization of the polylysine (DP = 190). The number of histidyl residues bonded per molecule of polylysine is x, and x = 70 in the preparation described above.

Figure 4

This shows the transfer of genes into HepG2 cells using polylysine (DP 190) partly substituted by histidyl residues (HispLK).

The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 µg in 0.7 ml DMEM) and the polylysine substituted by 70 histidyl residues (40 µg in 0.3 ml DMEM). After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up with 5% foetal bovine serum. The DNA/pLK complexes are formed by mixing the plasmid pCMVLUC (10 µg in 0.7 ml DMEM) and the polylysine (5 µg

in 0.3 ml DMEM). After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up with 5% foetal bovine serum and either with 100 μM chloroquine (+ chloro) or 20 μM of a fusiogenic peptide (+ E5CA) (GLFEAIAEFIEGGWEGLIEGCA). The medium in which the HepG2 cells (3 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution (1 ml) containing a DNA/polymer complex (5 μg/ml DNA). After incubation for 4 hours at 37°C, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds.

Under these conditions, 1 pg/ml luciferase produces 2,000 RLU.

From left to right on the abscissa axis, the first rectangle corresponds to the DNA/histidylated polylysine complex, the second rectangle corresponds to the DNA/polylysine complex, the third rectangle corresponds to the DNA/polylysine complex to which chloroquine is added and the fourth rectangle corresponds to the DNA/polylysine complex to which the fusiogenic peptide E5CA is added.

In the box: change in the efficiency of the transfection as a function of the amount of plasmid.

Figure 5

This relates to the transfer of genes into HepG2 cells using polylysine (DP 190) partly substituted by histidyl residues. It shows the influence of the number of histidyl residues bonded per molecule of polylysine on the efficiency of the transfection.

From left to right on the abscissa axis, the first rectangle corresponds to the DNA/unsubstituted polylysine complex, the second rectangle corresponds to the complex of DNA/polylysine substituted by 19 histidyl residues, the third rectangle corresponds to the complex of DNA/polylysine substituted by 30 histidyl residues, the fourth rectangle corresponds to the complex of DNA/polylysine substituted by 46 histidyl residues, the fifth rectangle corresponds to the complex of DNA/polylysine substituted by 63 histidyl residues, the sixth rectangle corresponds to the complex of DNA/polylysine substituted by 70 histidyl residues and the seventh rectangle corresponds to the complex of DNA/polylysine substituted by 84 histidyl residues.

The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 μg in 0.7 ml DMEM) and the polylysine substituted by a varying number of histidyl residues (40 μg in 0.3 ml DMEM). After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up with foetal bovine serum (final concentration 10%). The medium in which the HepG2 cells (3 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution (1 ml) containing a DNA/polymer complex (5 μg/ml DNA). After incubation for 4 hours at 37°C, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds.

Under these conditions, 1 pg/ml luciferase produces 2,000 RLU.

Figure 6

This relates to the transfer of genes into HOS cells using polylysine (DP 190) partly substituted by histidyl residues. It shows the influence of the DNA/polymer ratio (expressed on the abscissa in μg polymer per 100 μg DNA) in the pCMVLUC/His_{84}pLK complexes on the efficiency of the transfection.

The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 μg in 0.7 ml DMEM) and various amounts of polylysine substituted by 84 histidyl residues in 0.3 ml DMEM. After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up with 1% foetal bovine serum. The medium in which the HOS cells (2 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution (1 ml) containing a DNA/polymer complex (5 μg/ml DNA). After incubation at 37°C for 4 hours, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds.

Under these conditions, 1 pg/ml luciferase produces 2,000 RLU.

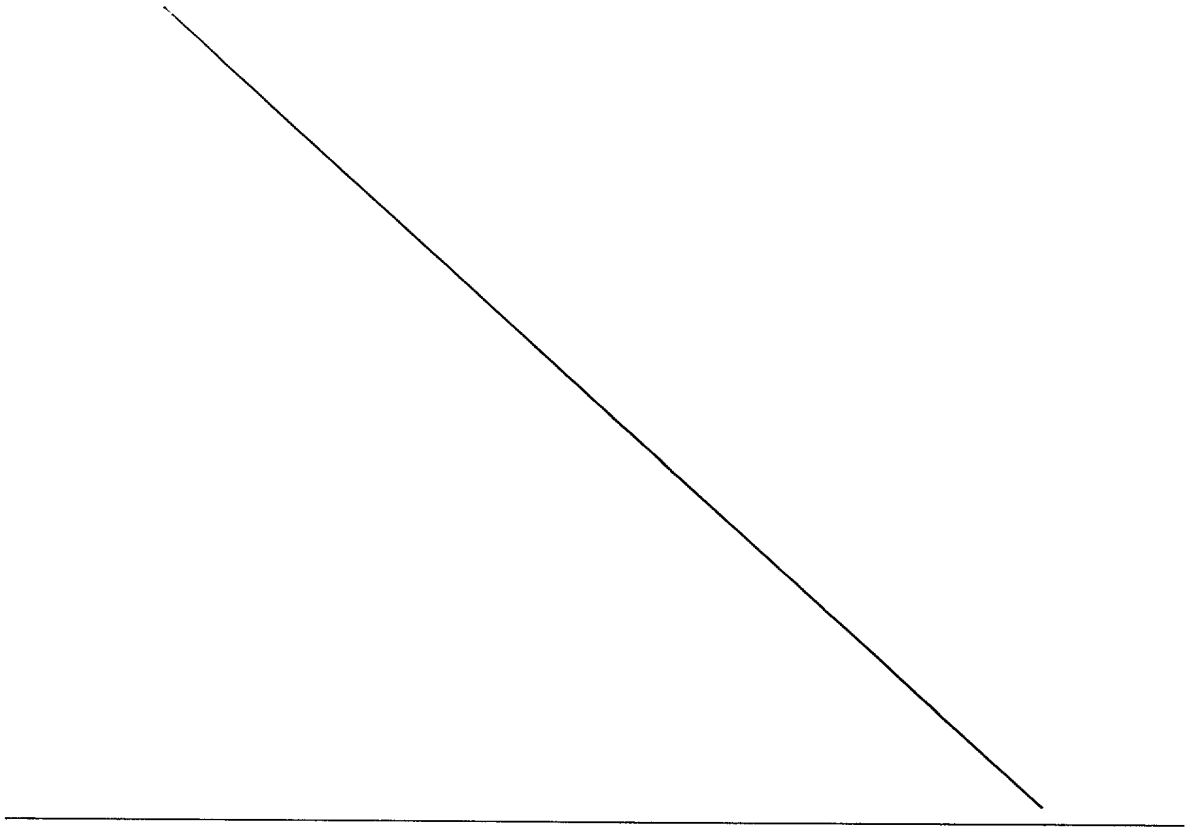


Figure 7

This relates to the transfer of genes into HepG2 cells using polylysine (DP 190) partly substituted by histidyl residues. It shows the influence of the incubation time (expressed in hours on the abscissa) of the pCMVLUC/His_{70}pLK complexes with the cells on the efficiency of the transfection. The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 µg in 0.7 ml DMEM) and polylysine substituted by 70 histidyl residues (40 mg in 0.3 ml DMEM). After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up to 10% with foetal bovine serum. The medium in which the HepG2 cells (3 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution (1 ml) containing a DNA/polymer complex (5 µg/ml DNA). After incubation at 37°C for various periods of time, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds.

Under these conditions, 1 pg/ml luciferase produces 2,000 RLU.

Figure 8

This relates to the transfer of genes into HepG2 cells using polylysine (DP 190) partly substituted by histidyl residues. It shows the influence of the amount of foetal bovine serum (expressed on the abscissa in % serum in the medium used) present during incubation of the pCMVLUC/His_{70}pLK complexes with the cells on the efficiency of the transfection. The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 µg in 0.7 ml DMEM) and polylysine substituted by 70 histidyl residues (40 ug in 0.3 ml DMEM). After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up with various amounts of foetal bovine serum. The medium in which the HepG2 cells (3 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution (1 ml) containing a DNA/polymer complex (5 µg/ml DNA). After incubation at 37°C for 4 hours, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds.

Under these conditions, 1 pg/ml luciferase produces 2,000 RLU.

Figure 9

This relates to the transfer of genes into various cell lines using polylysine (DP 190) substituted by 84 histidyl residues. The DNA/HispLK complexes are formed by mixing the plasmid pCMVLUC (10 µg in 0.7 ml DMEM) and polylysine substituted by 84 histidyl residues in 0.3 ml DMEM. After 30 minutes at 20°C, the solution containing the complexes is diluted once with DMEM and topped up to 10% with foetal bovine serum. The medium in which the cells (2-3 x 10^5 cells/4 cm^2) have grown for 24 hours is removed and replaced by a solution containing a DNA/polymer complex (5 µg/ml DNA). After incubation at 37°C for 4 hours, the cell medium is removed again and the cells are incubated in culture medium in the presence of 10% foetal bovine serum. The expression of the gene of luciferase was determined 48 hours after the transfection by measuring the luminescence emitted (RLU: relative values of the light emitted expressed in arbitrary units) in the cell lysates for 4 seconds. HepG2 = cell line derived from a human hepatocarcinoma; HOS = cell line derived

from a human osteosarcoma; MCF-7 = cell line derived from a human adenocarcinoma; B16 = cell line derived from a murine melanoma; COS = cell line derived from cells of the kidneys of the monkey transformed by SV40; Rb1 = cell line derived from smooth muscle cells of the aorta of the rabbit; HeLa = human epitheloid cell line; 16 HBE = epithelial cell line of the normal human respiratory tract; ΣCFTE = epithelial cell line of the human respiratory tract deficient with respect to the gene responsible for cystic fibrosis (CFTR).

Preparation of histidylated polylysine substituted by lactose

- Preparation of polylysine substituted by activated thiol groupings

Polylysine in the hydrobromide form (average molecular weight 40,000; average degree of polymerization 190) (1 g in 200 ml H_2O) originating from Bachem Feinchemikalien (Budendorf, Switzerland) is first passed over an anion exchange column (Dowex 2 x 8, OH^- form; 35 x 2.5 cm) in order to remove the bromide, which is toxic to cells. The polylysine solution is neutralized with a 10% solution of *p*-toluenesulphonic acid in water and then lyophilized.

The polylysine *p*-toluenesulphonate (50 mg; 0.91 μmole) is dissolved in 2 ml DMSO and reacted at 20ºC for 12 h with the N-hydroxysuccinimide ester of 4-carbonyl-α-methyl-α-(2-pyridinyldithio)toluene (SMPT, Pierce, USA) (5.3 mg; 13.6 μmoles). The polylysine substituted by carbonyl-α-methyl-α-(2-pyridinyldithio)toluene groups (= MPT-pLK) is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and recovered after renewed centrifugation. The residue is taken up in distilled water and the solution is lyophilized. The average number of MPT molecules bonded per molecule of polylysine is determined by the absorbance at 343 nm of pyridinethione (ε = 8,080 M^{-1} x cm^{-1}) released by quantitative reduction of the disulphide bond with the aid of TCEP (tris-carboxyethylphosphine): the average number of MPT per molecule of polylysine is 10.

- Preparation of histidylated polylysine substituted by activated thiol groupings.

Polylysine in the form of the *p*-toluenesulphonate substituted by 10 residues of MPT (50 mg; 0.96 μmole), dissolved in 3 ml DMSO (dimethylsulphoxide) in the presence of diisopropylethylamine (42 μl; 288 μmoles), is reacted for 24 hours at 20ºC with 32 mg (Boc)His(Boc)-OH (80 μmoles) in the presence of 43 mg benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (97 μmoles). The histidyl

residues are then deprotected in the presence of 20 ml of a mixture of water and trifluoroacetic acid (TFA) (50/50 V/V) to the extent of 50% for 24 hours at 20°C. The water and the TFA are removed by evaporation under reduced pressure. The polymer is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and collected after renewed centrifugation. The residue is taken up in distilled water and the solution is lyophilized. The number of histidyl residues bonded per molecule of polylysine, determined by proton NMR, is 60.

- *Reduction of dithiopyridyl*

The oligoside is first converted into the glycopeptide by a method described in French Patent Application 9407738 (Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]).

The glycopeptide is bonded to the partly histidylated polylysine via a disulphide bridge.

The Galβ4Glcβ-pyroglutamyl-NH-$(CH_2)_2$-S-S-pyridine (3 μmoles) is treated with 3.5 μmoles TCEP (tris-carboxyethylphosphine) in a sodium phosphate buffer, 0.1 M, at pH 7 (1 ml) for 1 h at 20°C. This solution is added to the partly histidylated polylysine substituted by 10 residues of MPT (10 mg; 0.2 μmole), dissolved in the sodium phosphate buffer, 0.1 M at pH 7 (1 ml). After 1 h at 20°C, the polymer is precipitated by addition of 10 volumes of isopropanol. The precipitate is collected after centrifugation (1,800 g, 15 min) and washed in isopropanol and then dissolved in water and lyophilized.

The yield of the coupling reaction under the conditions used is equal to or greater than 90%.

Preparation of histidylated polylysine substituted by a complex oligoside: Lewis^b

Example of Lewis^b = Fucα4(Fucα2Galβ3)GlcNAcβ3Galβ4Glc

Complex oligosides having a glucose (Glc) or N-acetylglucosamine (GlcNAc) residue in a reducing position are first converted into glycopeptides by a method described in French Patent Application 9407738 (Monsigny M., Sdiqui N., Roche A.C. and Mayer R., (1994) Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses]).

The complex oligosides are bonded to the partly histidylated polylysine by bonding of

the glycopeptide to the histidylated polylysine via a disulphide bridge.

The oligoside Fucα4(Fucα2Galβ3)GlcNAcβ3Galβ4Glc is converted into the glycopeptide derivative Fucα4(Fucα2Galβ3)GlcNAcβ3Galβ4Glcβ-pyroglutamyl-R. The carboxyl group of pyroglutamyl is substituted by a dithiopyridine function to give the glycopeptide: Fucα4(Fucα2Galβ3)GlcNAcβ3Galβ4Glcβ-pyroglutamyl-NH-$(CH_2)_2$-S-S-pyridine (French Patent Application 9407738: Monsigny M., Sdiqui N., Roche A.C. and Mayer R., 1994, Nouveaux dérivés d'oligosides, leur procédé de préparation et leurs applications [New derivatives of oligosides, a process for their preparation and their uses] and Quétard *et al.*, Simple synthesis of novel glycosynthons for glycoconjugate preparation: oligosylpyroglutamyl derivatives, in preparation).

- *Glycopeptide reduction*

The glycopeptide (2 µmoles) is treated with 2.2 µmoles TCEP (tris-carboxyethylphosphine) in a sodium phosphate buffer, 0.1 M at pH 7 (1 ml) for 1 h at 20°C. This solution is added to the partly histidylated polylysine substituted by 10 residues of MPT (10 mg; 0.2 µmole), dissolved in the sodium phosphate buffer, 0.1 M at pH 7 (1 ml). After 1 h at 20°C, the polymer is precipitated by addition of 10 volumes of isopropanol. The precipitate is collected after centrifugation (1,800 g, 15 min) and washed in isopropanol and then dissolved in water and lyophilized.

The yield of the coupling reaction under the conditions used is equal to or greater than 90%.

Preparation of histidylated polylysine substituted by the peptide ANP

- *Preparation of polylysine substituted by activated thiol groupings*

Polylysine in the hydrobromide form (average molecular weight 40,000; average degree of polymerization 190) (1 g in 200 ml H_2O) originating from Bachem Feinchemikalien (Budendorf, Switzerland) is first passed over an anion exchange column (Dowex 2 x 8, OH^- form; 35 x 2.5 cm) in order to remove the bromide, which is toxic to cells. The polylysine solution is neutralized with a 10% solution of *p*-toluenesulphonic acid in water and then lyophilized.

The polylysine *p*-toluenesulphonate (50 mg; 0.91 µmole) is dissolved in 2 ml DMSO and reacted at 20°C for 12 h with the N-hydroxysuccinimide ester of 4-carbonyl-α-methyl-α-(2-pyridinyldithio)toluene (SMPT, Pierce, USA) (5.3 mg; 13.6 µmoles). The polymer (MPT-

pLK) is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and recovered after renewed centrifugation. The residue is taken up in distilled water and the solution is lyophilized. The average number of MPT molecules bonded per molecule of polylysine is determined by the absorbance at 343 nm of pyridinethione (ε = 8,080 M^{-1} x cm^{-1}) released by quantitative reduction of the disulphide bond with the aid of TCEP: the average number of MPT is 10.

- Preparation of histidylated polylysine substituted by activated thiol groupings.

Polylysine in the form of the *p*-toluenesulphonate substituted by 10 residues of MPT (50 mg; 0.96 µmole), dissolved in 3 ml DMSO (dimethylsulphoxide) in the presence of diisopropylethylamine (42 µl; 288 µmoles), is reacted for 24 hours at 20°C with 32 mg (Boc)His(Boc)-OH (80 µmoles) in the presence of 43 mg benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (97 µmoles). The histidyl residues are then deprotected in the presence of 20 ml of a 50% solution of trifluoroacetic acid (TFA) for 48 hours at 20°C. The water and the TFA are removed by evaporation under reduced pressure. The polymer is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and collected after renewed centrifugation. The residue is taken up in distilled water and the solution is lyophilized. The number x of histidyl residues bonded per molecule of polylysine, determined by proton NMR, is 60.

- Reduction of the peptide ANP

The peptide ANP (CYSLRRSSAFGGRIDRIGAQSA) with its cysteine in the N-terminal position protected in the form of thiopyridinyl (7.5 mg; 2 µmoles) is reacted at 20°C for 15 minutes with TCEP (0.7 mg; 2 µmoles) in 1 ml of buffer, 0.1 M NaCl, 0.1 M tris/HCl pH 7.6.

- Preparation of histidylated polylysine substituted with the peptide ANP

The partly histidylated polylysine substituted by 10 molecules of MPT (MPT_{10}-,His_{70}pLK (10 mg; 0.2 µmole) in 1 ml of buffer, 0.1 M NaCl, 0.1 M tris/HCl pH 7.6, is reacted at 20°C for 24 hours with 7.5 mg (2 µmoles) of peptide ANP, the cysteine of which has been reduced. The polymer (ANP-S-,His_{70}-pLK) is precipitated by adding 10 volumes of isopropanol. After centrifugation (1,800 g x 15 minutes), the residue is washed with isopropanol and collected after renewed centrifugation. The residue is taken up in distilled

water and the solution is lyophilized. The average number of molecules of peptide ANP bonded per molecule of polymer is determined by analysis of the amino acids of the polymer by high pressure chromatography (HPLC) with a C_{18} reversed phase column (Supelcosil LC-18-DB, Supelco, Bellefonte, PA, USA), after hydrolysis of the polymer in HCl 5.6 N at 105ºC for 72 hours and conversion of the amino acids liberated into phenylthiohydantoin derivatives (PTH-aa). The average number of ANP per molecule of polymer is 8.

Preparation of histidylated polylysine substituted by biotin

Polylysine substituted by 60 histidyl residues (15 mg; 0.28 μmole), dissolved in 1 ml DMSO in the presence of DIEA (4 μl; 28 μmoles) is reacted for 7 h at 20ºC with the N-hydroxysuccinimide ester of 6-(biotinamido)hexanoate (NHS-LC-biotin, Pierce, USA). The polymer is precipitated by addition of 10 volumes of isopropanol. The precipitate is collected after centrifugation (1,800 g, 15 min) and washed in isopropanol and then dissolved in water and lyophilized.

CLAIMS

1. Complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, and/or of endosomes in a weakly acid medium,

- the abovementioned residues also having the following properties:

  . they carry a functional group which enables them to be bonded to the abovementioned polymer,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

  . they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

  . they carry at least one hydroxyl group,

  . they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

  . by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

  . on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), in particular on the hydroxyl groups of the abovementioned non-charged residues, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

2. Complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles and/or of endosomes, in a weakly acid medium,

- the abovementioned residues also having the following properties:

. they are bases of which the pK in an aqueous medium is less than 8, such that a proportion greater than 50% of these bases bonded to a cationic polymer is not protonated in a neutral medium of pH 7.4,

. they carry a functional group which enables them to be bonded to the abovementioned polymer,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

. they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

. they carry at least one hydroxyl group,

. they are not active with respect to the recognition signal recognized by a cell

membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

3. Complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, in a weakly acid medium,

- the abovementioned residues also having the following properties:

. they belong to the family of compounds which carry an imidazole nucleus,

. they belong to the family of quinolines,

. they belong to the family of pterines,

. they belong to the family of pyridines,

. the abovementioned residues carry a functional group which enables them to be bonded to the abovementioned polymer,

. they can carry at least one free NH_3^+ function,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex, with the proviso that all the abovementioned residues contain at least 30% of free NH_3^+ functions,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

. they carry at least one hydroxyl group,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysine), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

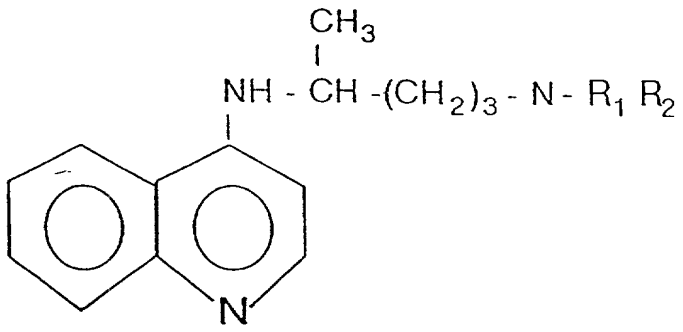
with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

4. Complex according to one of claims 1 to 3, in which the residues causing

destabilization of cell membranes in a weakly acid medium are

- alkylimidazoles in which the alkyl radical contains 1 to 10, in particular 2 to 6 carbon atoms, and in which only one of the nitrogen atoms of the imidazole nucleus is substituted,

- or quinolines of the formula:



in which R_1 represents H and R_2 represents $(CH_2)_n$-CO_2-H, n being an integer varying from 1 to 10, and preferably having a value of 1 to 3.

5. Complex according to any one of claims 1 to 3,

in which the residues causing destabilization of cell membranes are chosen from: histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(3-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl)ethylamine, β-alanyl-histidine-(carnosine), 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid,

and in which

- the optional free NH_3^+ function of the abovementioned residues (for example histidine) can also be substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of

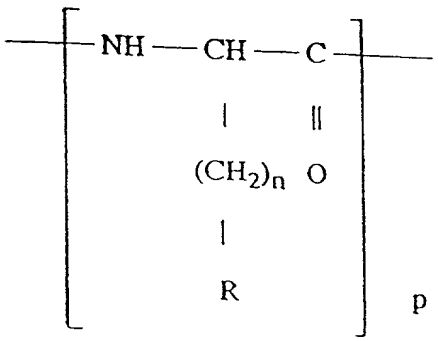
monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

6. Complex according to one of claims 1 to 5 between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, in particular residues of lysine or ornithine, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, by residues causing a destabilization of cell membranes in a weakly acid medium,
- the abovementioned residues also having the following properties:
  . they carry an imidazole nucleus,
  . they can carry at least one free NH_3^+ function,
  . they are not active with respect to the recognition signal,
- the remaining free NH_3^+ functions of the abovementioned monomer units also being substituted to the extent of about 1% to about 60% by a molecule which constitutes a recognition signal recognized by a cell membrane receptor, this recognition signal having a molecular weight of less than 5,000, and it being possible for this recognition signal to be present in an amount of one molecule for about 200 units of polymeric conjugate or about 60 molecules for about 200 units of polymeric conjugate,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

7. Complex according to one of claims 1 to 3, in which the polymer contains a polymeric grouping of the following formula (I):

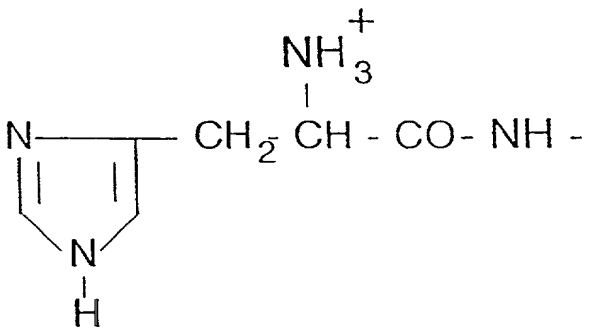


(I)

in which:

- p is an integer varying from 15 to 900, preferably 100 to 300,
- n is an integer varying from 1 to 6, and preferably has the value 4,
- this polymeric grouping contains radicals R among which:

. 10% to 45% of the number of radicals R representing a residue carrying an imidazole nucleus and optionally a free NH_3^+ function, in particular a histidyl residue, it being possible for R to be represented by the formula:



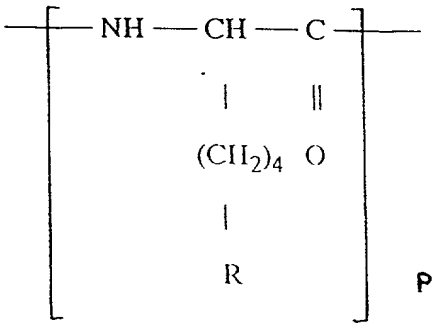
it being possible for the optional NH_3^+ function of the abovementioned residues also to be substituted by a molecule which constitutes a recognition signal,

10% to 90% of the number of radicals R representing free ϖ-amino NH_3^+ and optionally being substituted to the extent of 0 to 50% by a molecule which constitutes a recognition signal, in particular to the extent of 0 to 60, advantageously 1 molecule for about 200 units, or to the extent of 2 to 100, advantageously 50 molecules for about 200 units, and/or

it also being possible for R to be made up to the extent of 0 to 45% of a group $NH\text{-}CO\text{-}(CHOH)_m\text{-}R_1$, in particular a dihydroxypropionylamido, erythronylamido, threonylamido, ribonylamido, arabinylamido, xylonylamido, lyxonylamido, gluconylamido, galactonylamido, mannonylamido, glycoheptonylamido or glycooctonylamido radical, m is an integer from 2 to 15, preferably 2 to 7, R_1 represents H or an alkyl radical having 1 to 15 carbon atoms, in particular CH_3, it being possible for these radicals to be substituted by a molecule which constitutes a recognition signal, with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of

the abovementioned polymeric conjugate.

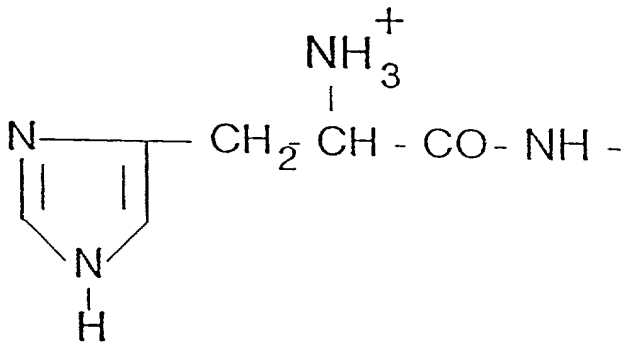
8. Complex according to claim 4, in which the polymer comprises a polymeric grouping of the following formula (II):



in which:

- p has the meanings indicated in claim 4,

- 10% to 45% of the number of radicals R represent a residue carrying an imidazole nucleus and optionally a free NH_3^+ function, in particular a histidyl residue, it being possible for R to be represented by the formula



it being possible for the NH_3^+ functions of the abovementioned residues also to be substituted by a molecule which constitutes a recognition signal,

- the remainder of the radicals, that is to say 30% to 90% of the number of radicals R,

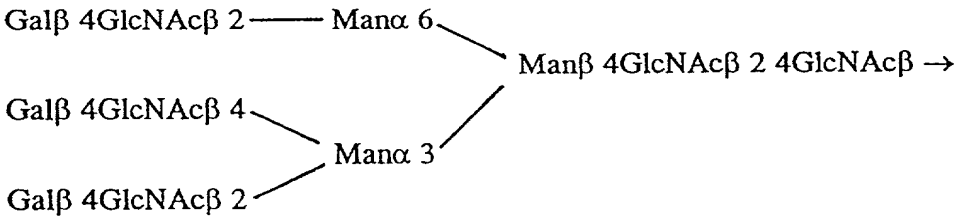
representing ϖ-amino NH_3^+, and it being possible for 0 to 45% of the radicals R to be substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

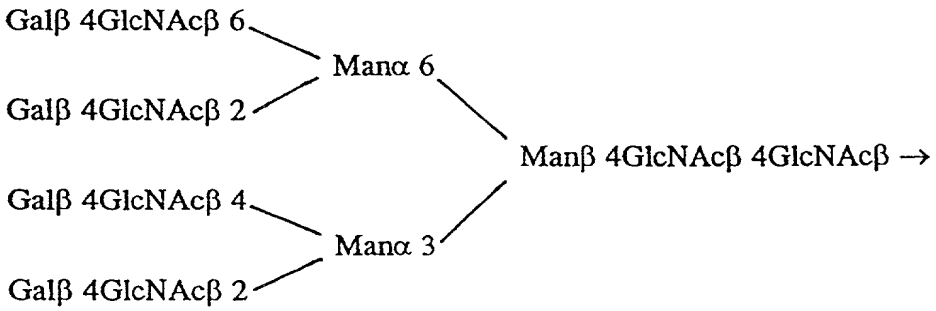
9. Complex according to one of claims 1 to 5, characterized in that the recognition signal is chosen from:

A) - simple or complex osides recognized by membrane lectins and chosen from:

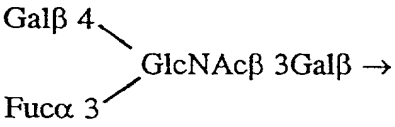
a.Asialo-oligoside of the type of triantennar lactosamine: asialoglycoprotein receptor



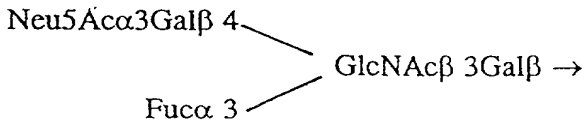
b. Asialo-oligoside of the type of tetraantennar lactosamine: asialoglycoprotein receptor



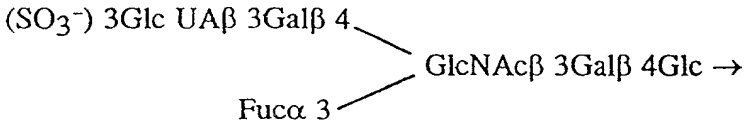
c. Lewis x: LECAM 2/3



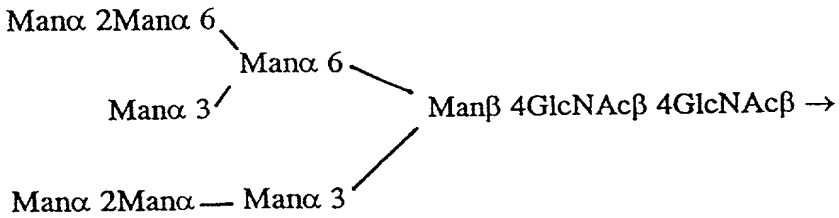
d. Lewis x sialyl: LECAM 3/2



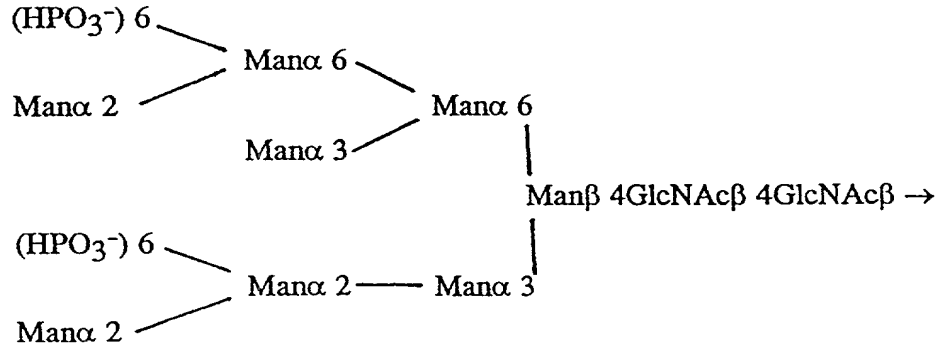
e. Sulphated Lewis x derivative (HNK1): LECAM 1



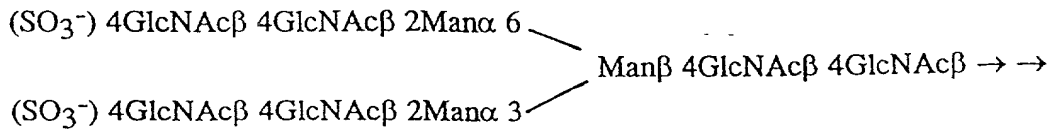
f. Oligomannoside: mannose receptor



g. Phosphorylated oligomannoside: mannose 6-phosphate receptor



h. Oligosaccharide of the type of sulphated lactosamine: sulphated GalNAc 4 receptor



B) Peptides

a) anti-inflammatory peptides or certain of their fragments recognized by receptors of the vascular wall, such as

- vasodilator intestinal polypeptide (VIP)

HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2

- atrial natriuretic polypeptide (ANP)

SLRRSSCFGGRMDRIGAQSGLGCNSFRY

- lipocortin

HDMNKVLDL

- bradykinin

RPPGFSPFR;

b) ligand peptides of integrins, such as peptides containing the sequence RGD, fibronectin ligand;

c) chemiotactic factors, such as formyl-peptides and their antagonists: FMLP, (N-formyl-Met-Leu-Phe);

d) peptide hormones, such as

α-MSH: Ac-SYSMEHFRWGKPV-NH_2 and their antagonists.

C) Natural metabolites, such as:

- biotin,

- carnitine,

- tetrahydrofolate and folic acid, which can be both a recognition signal with respect to certain cells having suitable receptors and a destabilizer of cell membranes.

10. Complex according to one of claims 1 to 6, characterized in that the nucleic acid can be chosen from:

a) marker genes, such as

- genes containing luciferase,

- green protein of the jellyfish *Aequarea victoria*,

- genes containing β-galactosidase,

- genes containing chloramphenicol acetyltransferase,

- genes which confer resistance to an antibiotic, such as hygromycin, neomycin etc....;

b) genes with a therapeutic purpose, such as

- receptors of lipoproteins of low-density, which are deficient in cases of hypercholesterolaemia,

- coagulation factors: factors VIII and IX,

- phenylalanine hydroxylase (phenylketonuria),

- adenosine deaminase (ADA immunodeficiency),

- lysosomal enzymes, such as β-glucosidase in the case of Gaucher's disease,

- dystrophin and minidistriphin (myopathy),

- tyrosine hydroxylase (Parkinson),

- neurone growth factors (Alzheimer),

- CFTR cystic fibrosis transmembrane conductance regulator (cystic fibrosis),

- alpha-1-antitrypsin,

- cytokines (interleukins, TNF tumour necrosing factor),

- thymidine kinase of the Herpes simplex virus,

- proteins of MHC, major histocompatibility complex, in particular HLA-B7,

- cytosine deaminase,

- genes which code for sense and antisense RNAs,

- genes which code for ribozymes,

c) genes for the purpose of vaccines

- genes which code for viral antigens (vaccination), for example: the gene which codes for the nucleoprotein of the influenza virus.

11. Complex according to one of claims 1 to 7, in which:

- the polymer, in particular polylysine, has a degree of polymerization of about 15 to about 900, preferably 200,

- the free NH_3^+ functions of the lysine units being substituted in a ratio of 35% by histidyl residues and optionally by a molecule which constitutes a recognition signal for 1 to 50 residues of lysine, where the said signal molecule has an affinity of at least 10^5 l $mole^{-1}$ with respect to the receptor of the cell which the complex is to target, or optionally by 20 to 100 molecules of recognition signal for 200 lysine residues, where the said signal molecule

has an affinity of less than 10^5 l $mole^{-1}$ with respect to the said receptor,

- the nucleic acid has a molecular weight of about 10^6 to about 10^8, in particular 3.10^6 to 30.10^6,

- the ratio between the average number of base pairs of the nucleic acid per molecule of monomer unit, in particular lysine, is about 0.2 to about 6, preferably about 0.4 to about 0.6.

12. Positively charged polymeric conjugate containing units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10%, advantageously about 15% to about 45%, in particular 35%, this ratio being determined, for example, by nuclear magnetic resonance, by residues causing a destabilization of cell membranes, in particular the membrane of endocytosis vesicles, in a weakly acid medium,

- the abovementioned residues also having the following properties:

. they carry a functional group which enables them to be bonded to the abovementioned polymer,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

. they can carry at least one free NH_3^+ function,

- it being possible for the free NH_3^+ functions of the abovementioned monomer units also to be substituted by non-charged residues causing a reduction in the positive charges with respect to the same unsubstituted polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex,

- the abovementioned non-charged residues also having the following properties:

. they carry at least one hydroxyl group,

. they are not active with respect to the recognition signal recognized by a cell membrane receptor,

. it being possible for the hydroxyl groups of the abovementioned non-charged residues to be substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor,

- molecules constituting a recognition signal recognized by a cell membrane receptor optionally being present:

. by substitution of some of the free NH_3^+ functions of the abovementioned monomer units (for example ε-NH_3^+ of lysines), or

. on some of the abovementioned non-charged residues causing a reduction in the charge (for example gluconyl), and in particular on the hydroxyl groups of the abovementioned non-charged residues causing a reduction in charge, or

. on some of the abovementioned residues causing a destabilization of cell membranes (for example acetylimidazole), or

. by substitution of the optional free NH_3^+ function of the abovementioned residues causing a destabilization of cell membranes (for example histidine),

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

13. Polymeric conjugate according to claim 12 and as defined according to one of claims 2 or 3, or containing a polymeric grouping of the formula according to one of claims 4 or 5,

14. Use of a complex according to one of claims 1 to 11 or a conjugate according to one of claims 12 or 13 for the *in vitro, ex vivo* or *in vivo* transfection of cells with the aid of a gene, in particular those defined in claim 6.

15. Use of a complex or a conjugate according to claim 11, characterized in that the cells are chosen from;

- cells of haematopoietic strains;
- dendritic cells;
- liver cells;
- skeletal muscle cells;
- skin cells:
  - . fibroblasts,
  - . keratinocytes,
  - . dendritic cells,

. melanocytes;

- cells of the vascular walls;

. endothelial;

. smooth muscle;

- epithelial cells of the respiratory tract;

- cells of the central nervous system;

- cancerous cells;

- cells of the immune system, such as lymphocytes, macrophages, NK cells etc....

16. Method of *in vitro* or *ex vivo* transfection, characterized in that a complex according to any one of claims 1 to 11 is brought into contact with a medium containing cells to be transfected under conditions such that there is:

- passage of the complex from the medium into the cytoplasm of the cells,

- salting out of the nucleic acid involved in the abovementioned complex in the cytosol and/or the nucleus of the cells,

- transcription and expression of the nucleic acid in the transfected cells,

- expression of the protein corresponding to the transfected gene.

17. Pharmaceutical composition, characterized in that it comprises, as the active substance, at least one of the complexes according to any one of claims 1 to 11, or at least one of the conjugates according to one of claims 12 or 13, in combination with a pharmaceutically acceptable vehicle.

18. Use of a complex according to one of claims 1 to 11 or a conjugate according to one of claims 12 or 13 for the preparation of a medicament intended, for example, for treatment of congenital or acquired metabolic deficiency, or treatment of tumours, or for the preparation of a vaccine, for example a vaccine against influenza.

19. Set or kit comprising:

- a polymeric conjugate according to one of claims 12 or 13, such as polylysine substituted by a residue causing a destabilization of cell membranes in a weakly acid

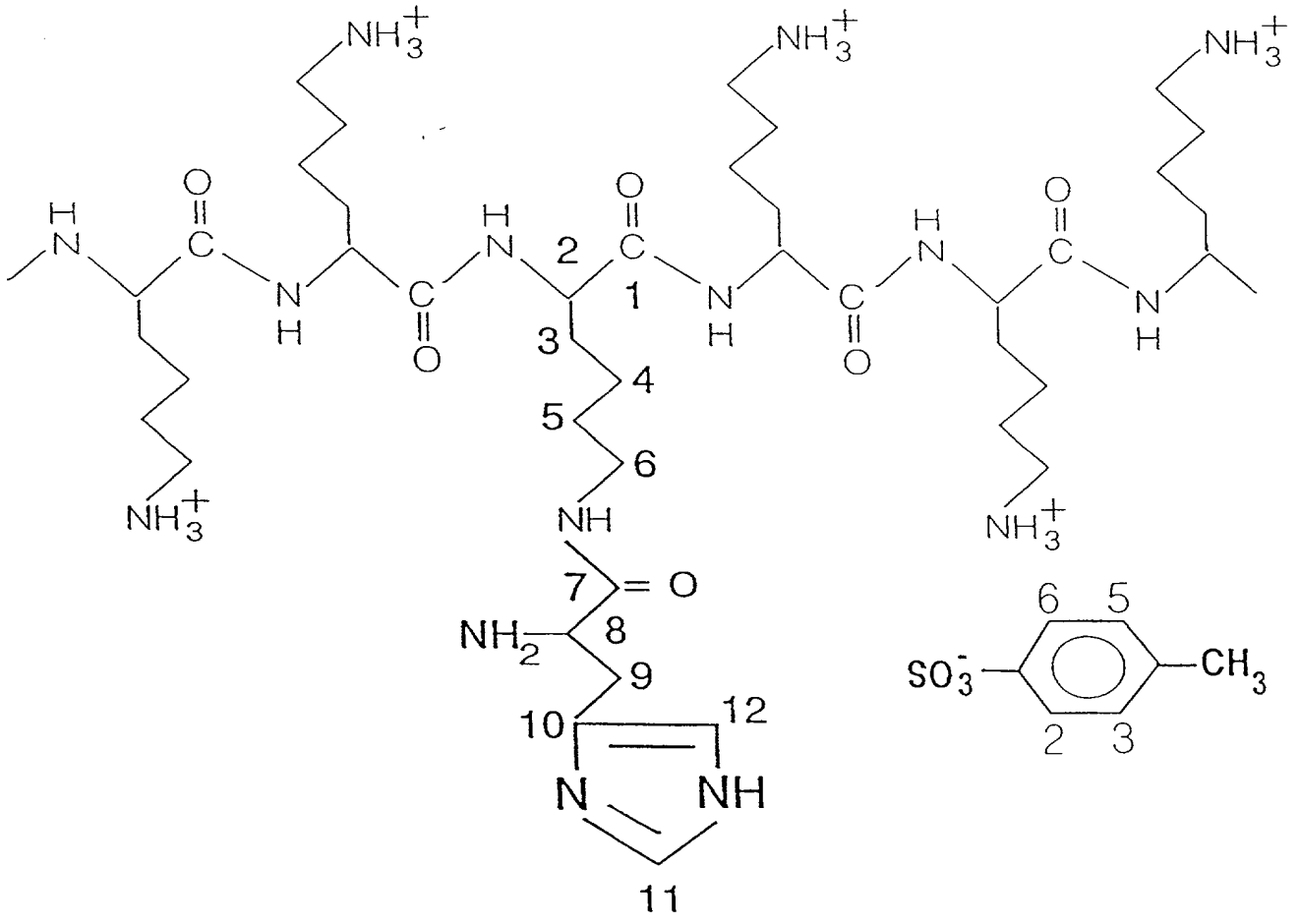
medium, this polymeric conjugate being capable of optionally carrying a recognition signal, which is or is not bonded beforehand to the abovementioned polymeric conjugate, the said recognition signal being a function of the cell to be targeted,

- optionally a plasmid containing at least one gene to be transferred, and optionally the system for regulation of the expression of the abovementioned gene,

- reagents which allow optional bonding of the recognition signal on to the abovementioned polymeric conjugate,

- reagents which allow the formation of a complex according to one of claims 1 to 11, or between the polymeric conjugate and the gene to be transferred, or between the polymeric conjugate and a plasmid containing the gene to be transferred,

- reagents which allow transfection of the cell by the abovementioned complex.



HispLK

Figure 1

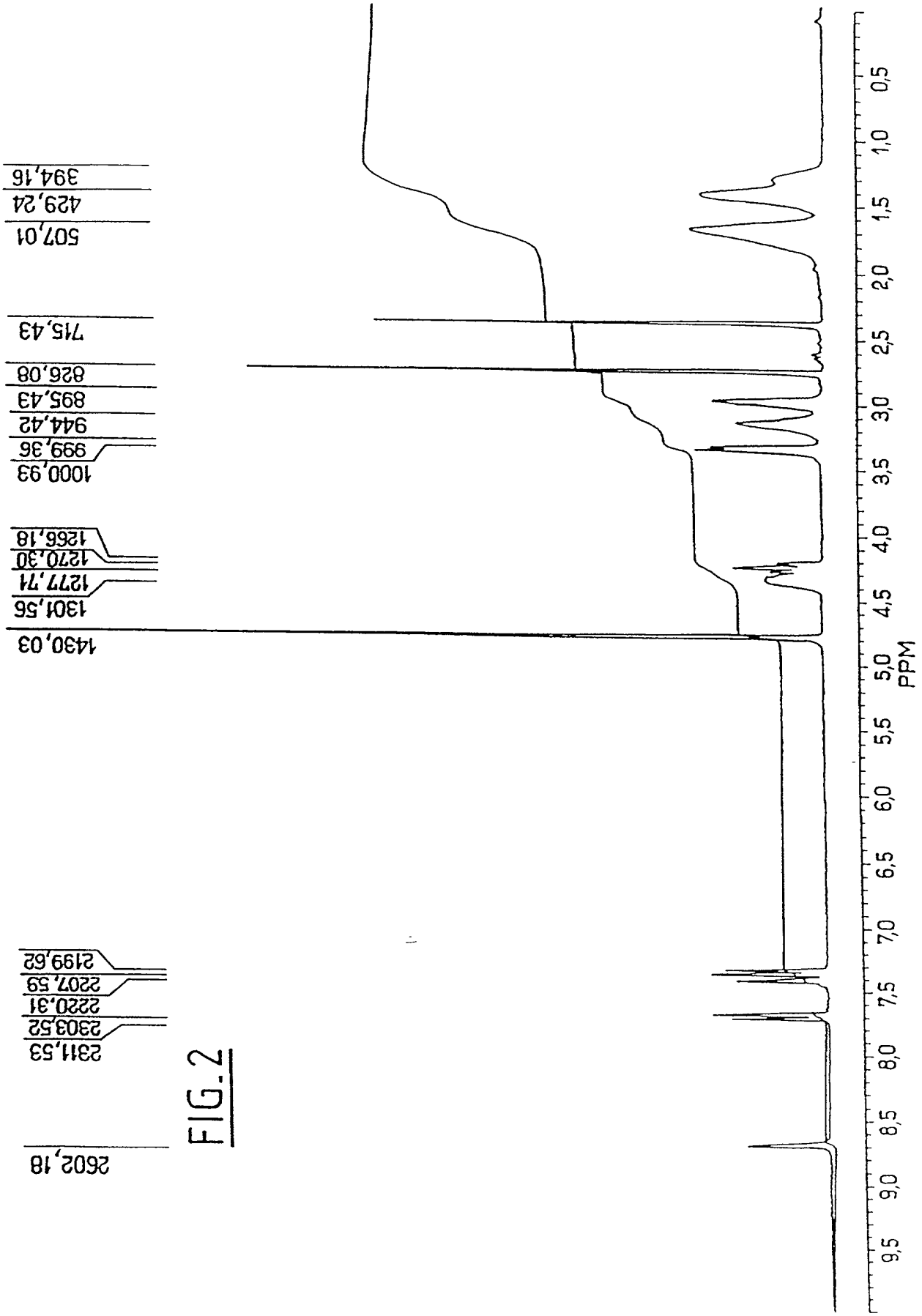

2602,18
2311,53
2303,52
2220,31
2207,59
2199,62
1430,03
1301,56
1277,71
1270,30
1266,18
1000,93
999,36
944,42
895,43
826,08
715,43
507,01
429,24
394,16
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0
PPM
5,5
6,0
6,5
7,0
7,5
8,0
8,5
9,0
9,5

FIG.2

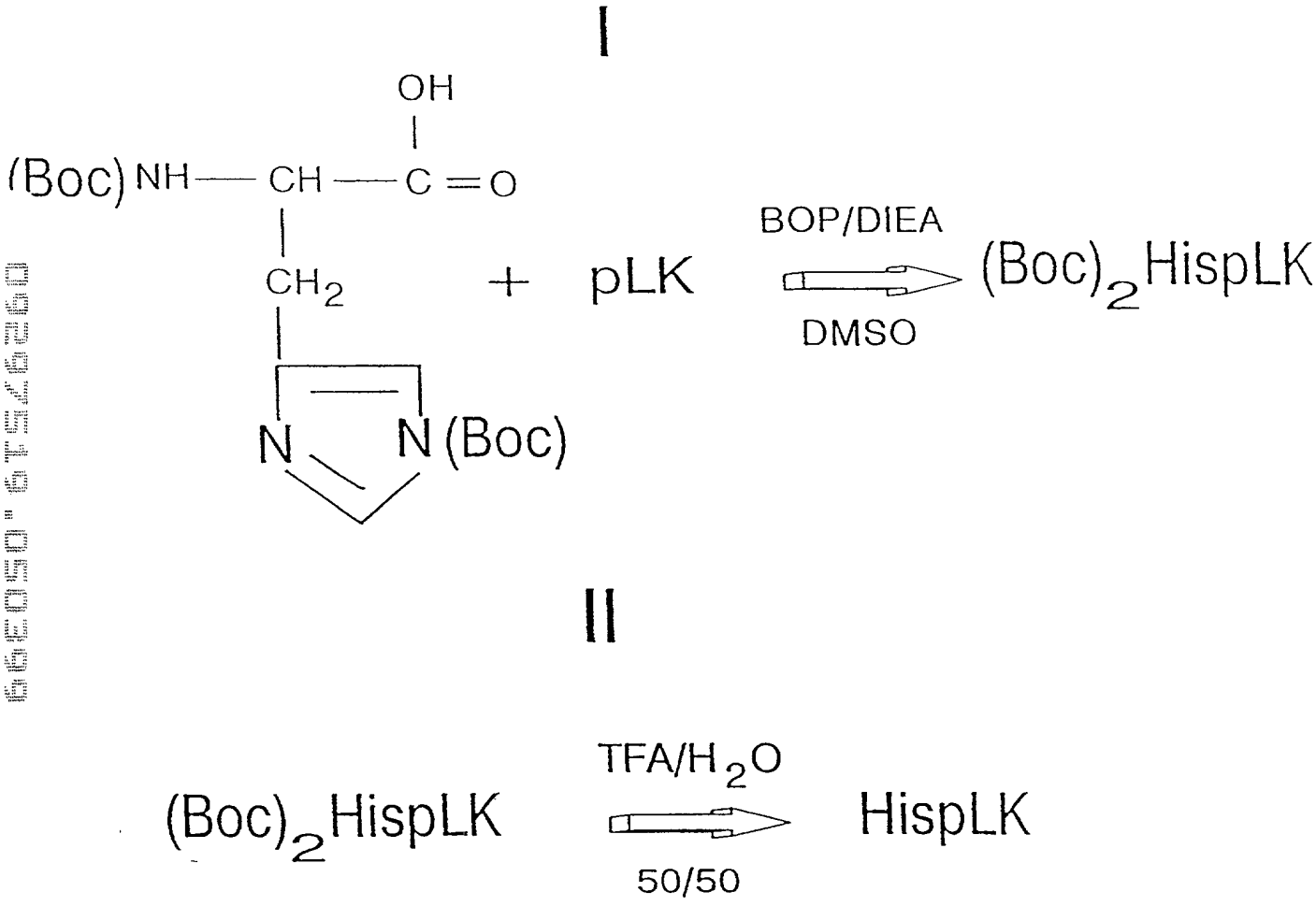

I
OH
(Boc) NH — CH — C = O
CH2
+ pLK
BOP/DIEA
DMSO
(Boc)2HispLK
N
N (Boc)
II
TFA/H2O
(Boc)2HispLK
50/50
HispLK

Figure 3

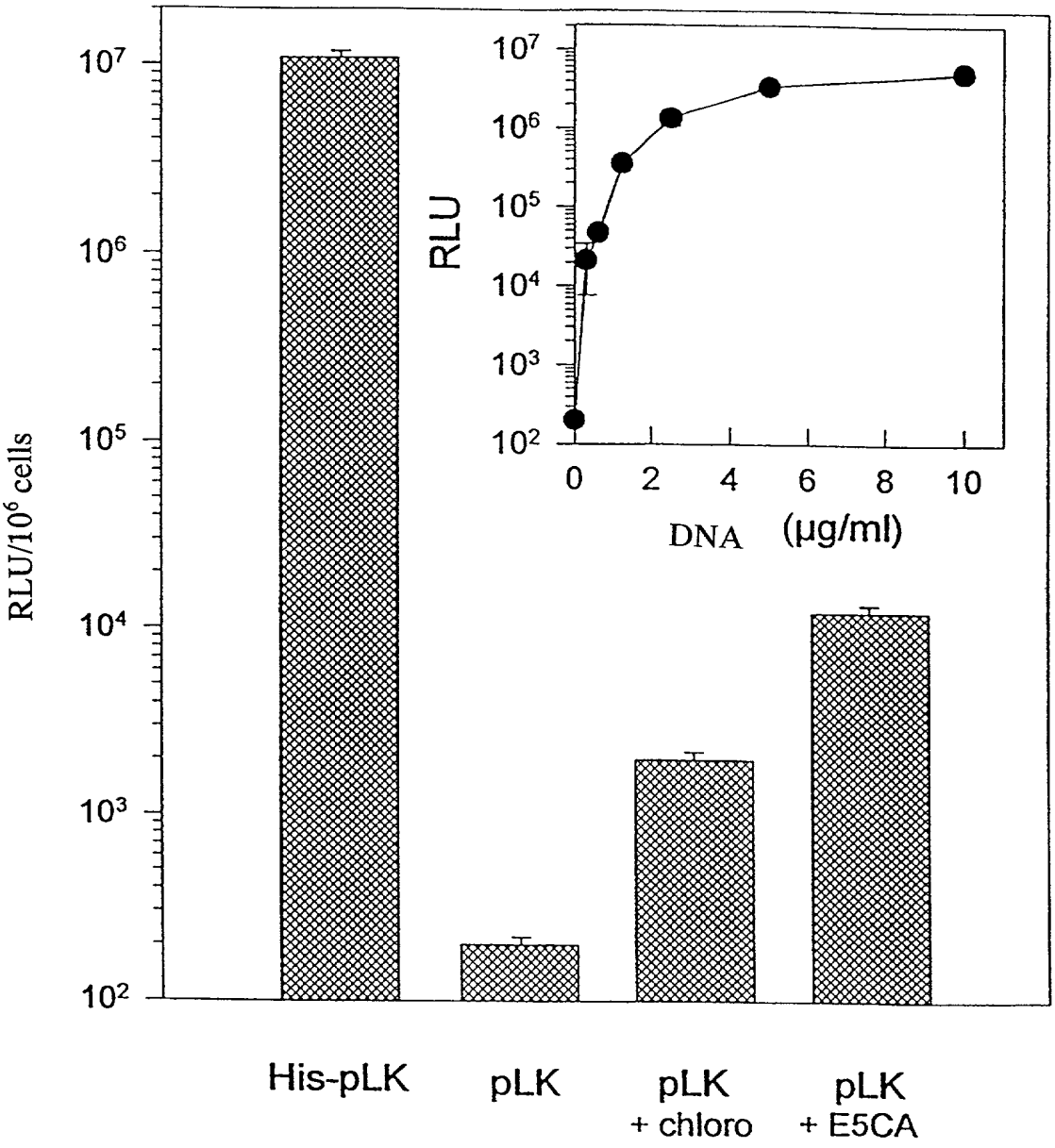

RLU/10^6 cells
10^7
10^6
10^5
10^4
10^3
10^2
RLU
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
DNA (µg/ml)
His-pLK
pLK
pLK + chloro
pLK + E5CA

Figure 4

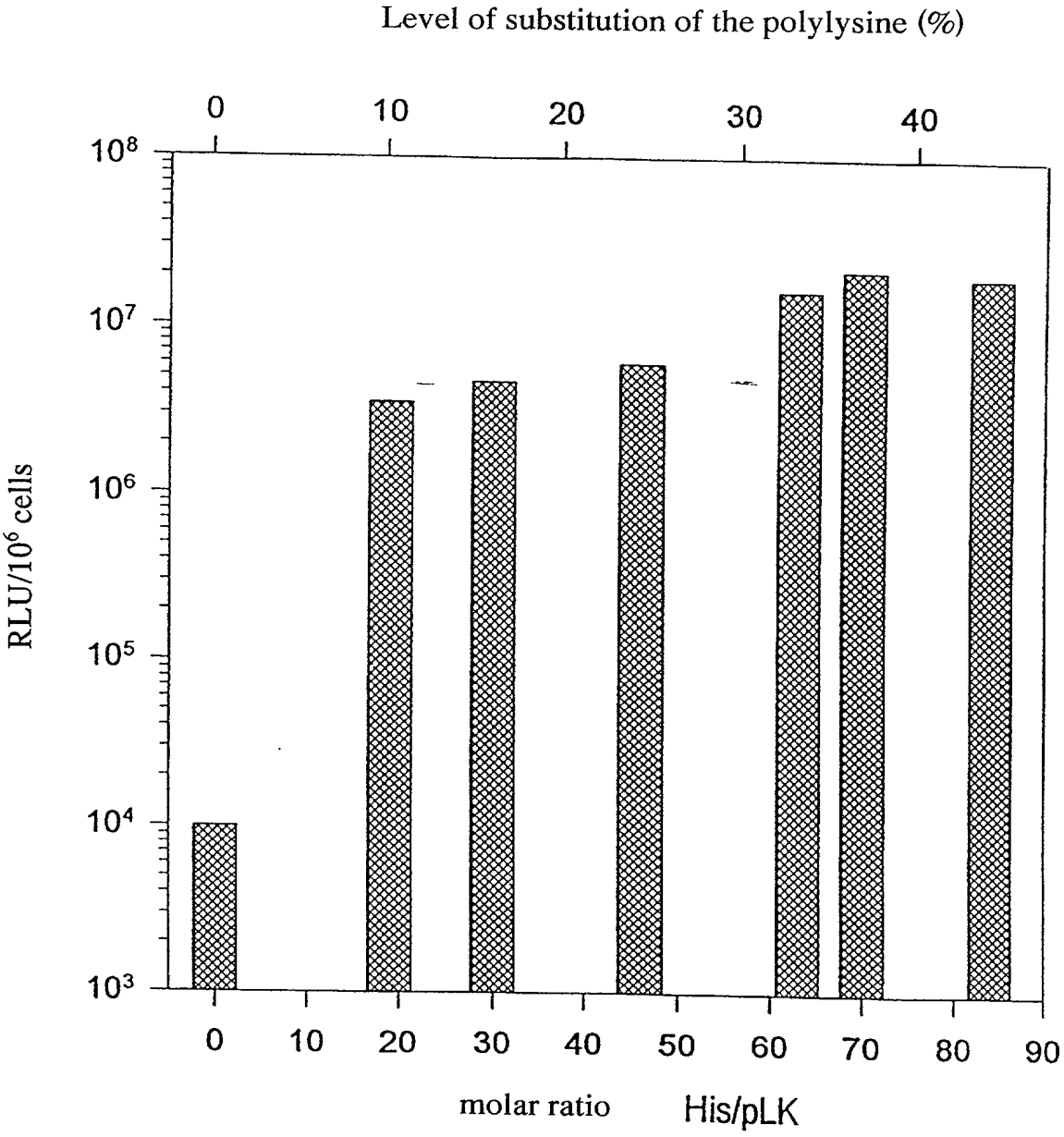

Level of substitution of the polylysine (%)
0
10
20
30
40
10^8
10^7
10^6
10^5
10^4
10^3
RLU/10^6 cells
0
10
20
30
40
50
60
70
80
90
molar ratio His/pLK

Figure 5

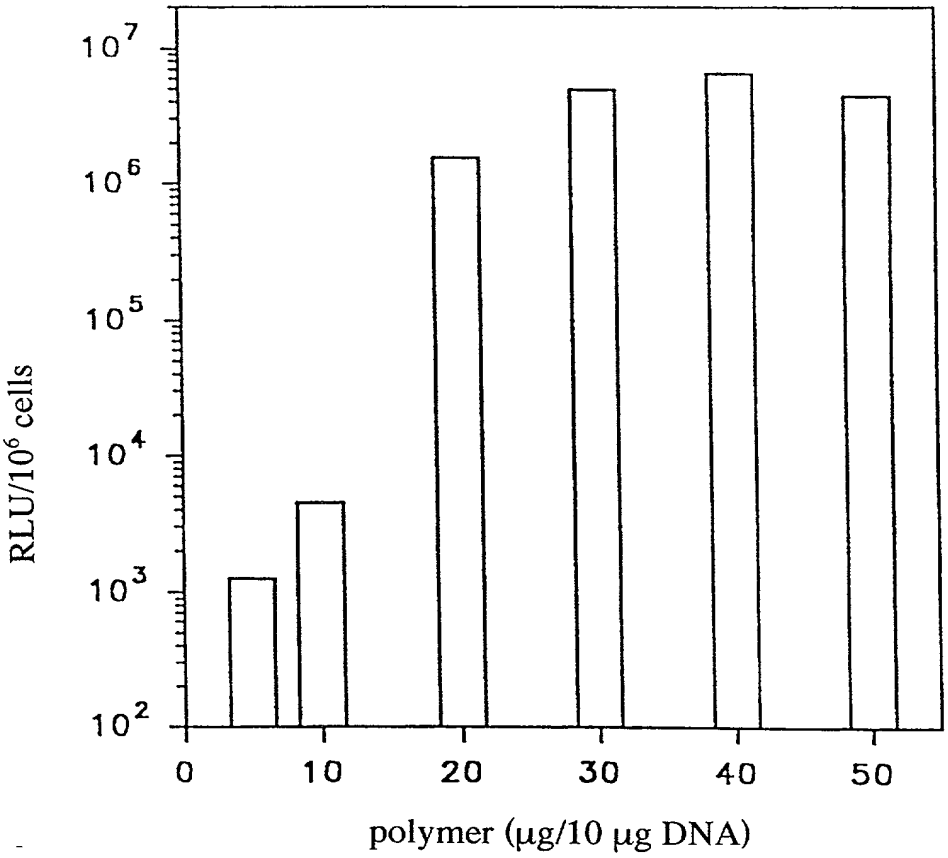

10^7
10^6
10^5
10^4
10^3
10^2
RLU/10^6 cells
0
10
20
30
40
50
polymer (µg/10 µg DNA)

Figure 6

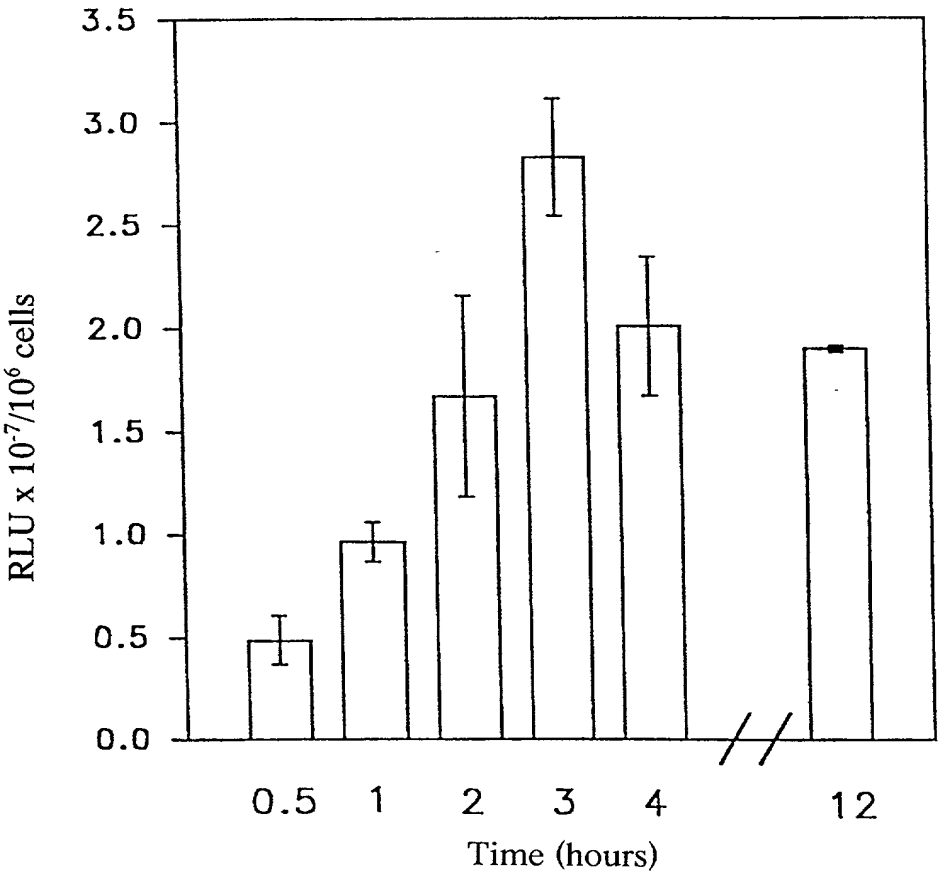


Figure 7

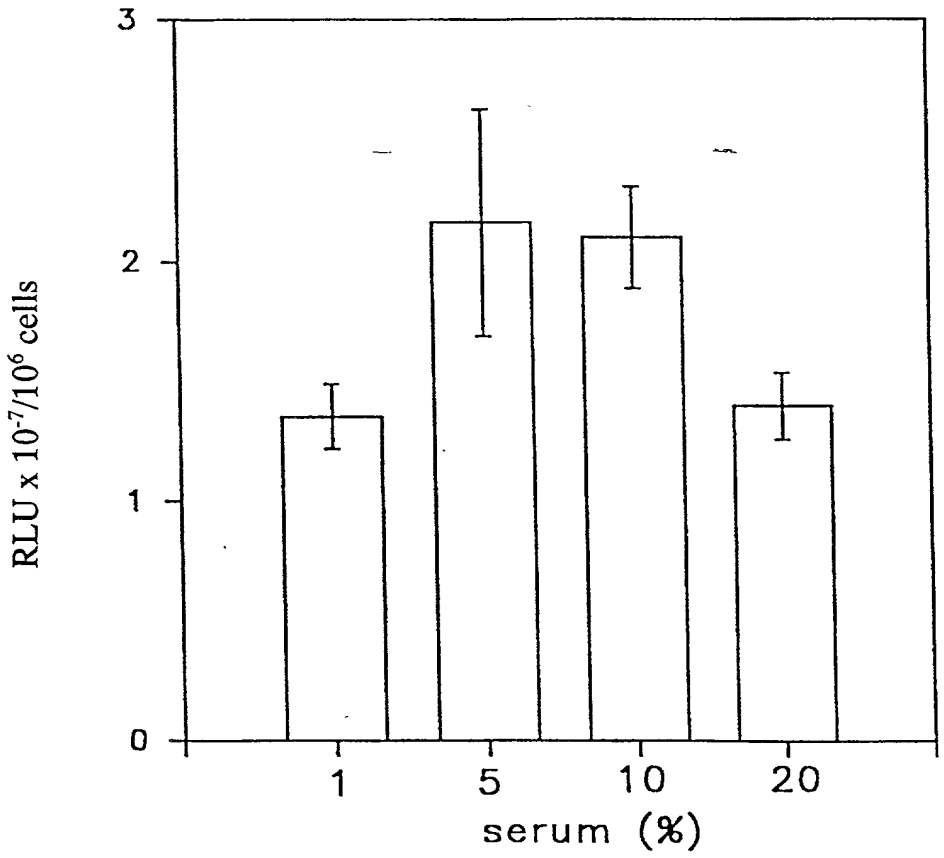


Figure 8

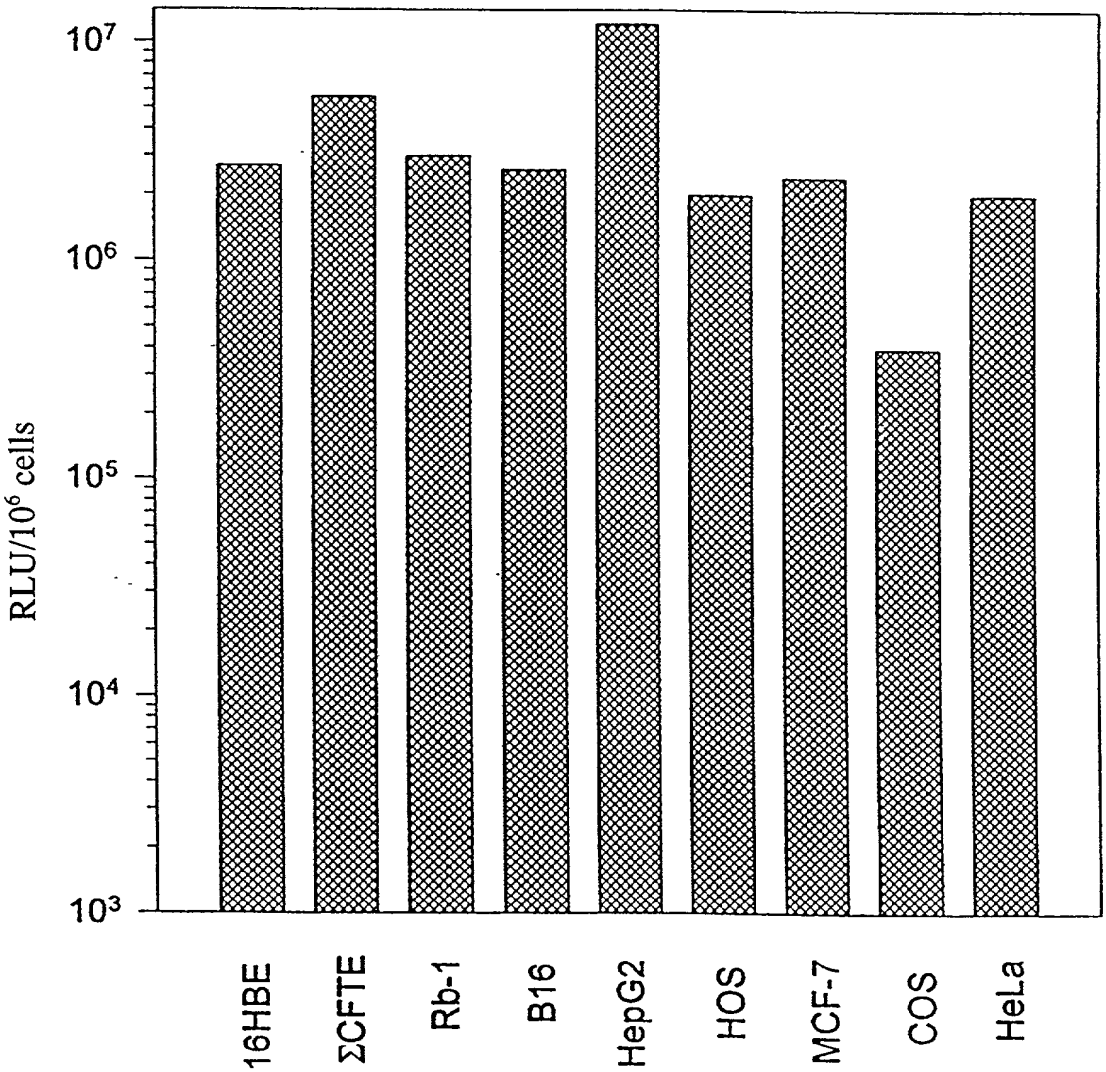

10^7
10^6
10^5
10^4
10^3
RLU/10^6 cells
16HBE
ΣCFTE
Rb-1
B16
HepG2
HOS
MCF-7
COS
HeLa

Figure 9

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (8-96)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

☒ Declaration Submitted with Initial Filing OR ☐ Declaration Submitted after Initial Filing

Attorney Docket Number	410.015
First Named Inventor	Patrick MIDOUX et al
COMPLETE IF KNOWN	
Application Number	PCT/FR97/02022
Filing Date	November 10, 1997
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

NOVEL POLYMERIC COMPLEXES FOR THE TRANSFECTION OF NUCLEIC ACIDS, WITH RESIDUES CAUSING THE DESTABILIZATION OF CELL MEMBRANES

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 11/10/97 as United States Application Number or PCT International Application Number PCT/FR97/02022 and was amended on (MM/DD/YYYY) _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	Certified Copy Attached? NO
96/13990	France	11/15/96	☐	☐	☒
PCT/FR97/02022	PCT	11/10/97	☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

US

Please type a plus sign (+) inside this box → +

Approved for use through 9/30/98 (0651-0031)
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Bierman, Muserlian and Lucas	18,818		
Jordan B. Bierman	18,629		
Charles A. Muserlian	19,683		
Donald C. Lucas	31,275		

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

Direct all correspondence to:

Name	Charles A. Muserlian				
Address	Bierman, Muserlian and Lucas				
Address	600 Third Avenue				
City	New York	State	NY	ZIP	10016
Country	U.S.A.	Telephone	(212) 661-8000	Fax	(212) 661-8002

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name	Middle Initial	Family Name	Suffix e.g. Jr.
Patrick		MITOUX	

Inventor's Signature: [signature] Date: 04/22/99

Residence: City	State	Country	Citizenship
Orleans FRX		France	France

Post Office Address	
Post Office Address	21, rue du Poinçon, F-45100 Orleans, France

City	State	ZIP	Country
Orleans		F-45100	France

☒ Additional inventors are being named on supplemental sheet(s) attached hereto

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (8-96)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number

DECLARATION

ADDITIONAL INVENTOR(S) Supplemental Sheet

2

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name	Michel	Middle Initial		Family Name	Monsigny	Suffix e.g. Jr.	
Inventor's Signature						Date	04/22/99
Residence: City	Saint-Cyr-En Val	State		Country	France FR	Citizenship	France
Post Office Address	341, rue des Bouvreuils, F-45590, Saint-Cyr-En-Val, France						
Post Office Address							
City	Saint-Cyr-En-Val	State		Zip	F-45590	Country	France

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name		Middle Initial		Family Name		Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip		Country	

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name		Middle Initial		Family Name		Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip		Country	

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name		Middle Initial		Family Name		Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip		Country	

☐ Additional inventors are being named on supplemental sheet(s) attached hereto

United States Patent & Trademark Office

Office of Initial Patent Examination – Scanning Division

Application deficiencies were found during scanning:

☐ Page(s) 4 of 5 - 5 of 5 of Declaration were not present for scanning. (Document title)

☐ Page(s)_____ of _____ were not present for scanning. (Document title)

☐ Scanned copy is best available.